Physiological Characterization of the Wheat ABC Transporter Lr34

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Summary

The resistance gene *Lr34res* from wheat confers partial, durable and broad-spectrum resistance against several biotrophic pathogens, such as leaf rust, stripe rust or powdery mildew. The gene encodes for a full-size ABC transporter of the G-subfamily, also known as pleiotropic drug resistance (PDR) transporters. Interestingly, the resistance is caused by only two mutations in the coding region of the original *Lr34sus* allele, which lead to a phenylalanine deletion and a tyrosine to histidine conversion in the first transmembrane domain of the transporter. When transferred into rice, sorghum, maize or barley, *Lr34res* can even confer resistance against species-specific pathogens like rice blast, barley powdery mildew and several rust species. However, a strong expression level of the transgene often causes side-effects, such as the induction of drought stress in rice, an accumulation of neutral lipids in barley leaves or the development of leaf tip necrosis as a sign of early senescence. The aim of this thesis was to investigate the molecular mechanism of the ABC transporter in an attempt to link this mechanism to the observed side-effects.

First, the subcellular localization of the ABC transporter was investigated. A fractionation of total membranes from tobacco BY2 cells expressing HA-tagged Lr34res protein showed that the transporter is located in the plasma membrane (PM), which is in agreement with the absence of a signal peptide and reports about the localization of related PDR transporters. This was the basis for functional studies with the aim to characterize the substrate spectrum of Lr34. It was assumed that the ABC transporter is involved in lipid metabolism because certain lipids play key roles as signaling molecules in biotic and abiotic stress responses and can regulate the activation of the corresponding signal transduction cascades. In addition, numerous studies have proven a direct role of ABC transporters – especially of the G-subfamily – in the translocation of polar lipids within the membrane and further studies could relate disorders in human lipid storage to the malfunction of certain ABC transporters. Finally, the transcriptomic changes in *Lr34res*-expressing barley and rice plants have revealed the upregulation of important genes involved in lipid metabolism, which for example results in triacylglycerol accumulation in barley leaves. Therefore, it was investigated whether Lr34 is able to translocate certain phospholipids (PLs) from one PM monolayer to the other, a process called PL flipping (exoplasmic to cytoplasmic leaflet) or PL flopping (cytoplasmic to exoplasmic leaflet). In a first approach *Lr34res*-expressing barley protoplasts were incubated with fluorescence-labelled PLs to observe their time-dependent incorporation. This experiment demonstrated that phosphatidic acid (PA) accumulated and phosphatidylserine (PS) was exported in the presence of Lr34res, while the uptake rates of phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were not affected. In order to confirm

these results, transgenic tobacco BY2 protoplasts were transfected with PL-specific biosensors for the visualization of the subcellular distribution of specific native PLs. With this approach a reduced PS content in the cytoplasmic PM leaflet of *Lr34res*-expressing protoplasts could indeed be confirmed. Interestingly, the level of phosphatidylinositol (4,5)-bisphosphate was also reduced in the presence of Lr34res. Finally, the ABC transporter was heterologously expressed in *Saccharomyces cerevisiae* to perform growth assays that use toxins to probe for the translocation of specific endogenous PLs at the PM. This experiment revealed an increased sensitivity to the PS-specific toxin papuamide A in *Lr34res*- and *Lr34sus*-expressing cells as a consequence of PS-exposure in the PM. Thus, not only Lr34res but also Lr34sus works as PS floppases. Both versions of the ABC transporter in addition decreased the sensitivity to the PE-specific toxin duramycin, pointing to a reduction of PE-exposure in the yeast PM, although this PL was not found to be a potential substrate in the PL translocation assay using barley protoplasts. In summary, all tested systems consistently confirmed the PS floppase activity of Lr34res (and Lr34sus) and could identify further potential PL substrates of the ABC transporter.

When heterologously expressed in a temperature sensitive yeast mutant defective in PL transfer Lr34res but not Lr34sus promoted the accumulation of neutral lipids in oil bodies, pointing to distinct activities of the two transporter versions. This observation is in agreement with the increased triacylglycerol content in leaves of *Lr34res*-expressing barley plants and supports the assumed role of Lr34res in overall cellular lipid metabolism.

As physiological stress responses are very often associated with an induction of PL-modifying enzymes, it was assumed that Lr34res might indirectly alter their activity. However, at least in transgenic barley no indications for an enhanced or decreased Lr34res-promoted turnover of the most abundant membrane PLs were found, suggesting that the observed stress responses are either caused by an indirect stimulation of the formation of low-abundant signaling lipids (polyphosphoinositides) or by the PL translocator activity of Lr34res *per se*.

For the characterization of the full substrate spectrum as well as the elucidation of putative differences in activity between the two Lr34 versions by using functional assays, the ABC transporters were heterologously expressed in yeast and in BY2 cells to produce large protein amounts for the consecutive purification and reconstitution in liposomes with a defined lipid composition. A comparison of the two expression systems revealed that a higher expression level of Lr34 was achieved in BY2 cells compared to yeast cells and in addition the protein could be efficiently solubilized from the membrane. However, although the HA-tagged Lr34 protein could be purified

from the solubilized microsomal fraction of large scale BY2 cultures, the amount was by far not sufficient for reconstitution into liposomes.

Finally, transcriptomic data from transgenic rice plants have shown that the ABC transporter is able to induce drought stress in rice (S. Krattinger, personal communication). Therefore, it was investigated if the upregulation of these drought stress-related genes goes along with a reduced transpiration in the leaves. In fact, gas exchange measurements demonstrated that not only the transpiration rate but also the photosynthetic activity were decreased due to reduced stomatal conductance in rice plants with a strong expression of *Lr34res*.

In summary, the ABC transporter Lr34 changes the distribution of certain PLs in the PM, which is thought to have severe consequences for the membrane associated proteome. More precisely, PS (and phosphatidylinositol (4,5)-bisphosphate)-binding proteins are expected to be detached from the PM while PA-binding proteins would get attracted. These changes presumably activate the expression of stress- and pathogenicity-related genes via specific signal transduction cascades resulting in the observed phenotypes in *Lr34res*-expressing plants, such as increased pathogen resistance, drought stress induction or neutral lipid accumulation.

Zusammenfassung

Das Resistenzgen *Lr34res* aus Weizen vermittelt partielle und dauerhafte Resistenz mit einem breiten Spektrum gegen diverse biotrophe Krankheitserreger, wie z.B. Braunrost, Gelbrost oder Mehltau. Das Gen kodiert für einen Volllänge-ABC-Transporter der G-Unterfamilie, die auch als PDR (pleiotropic drug resistance)-Transporter bekannt sind. Interessanterweise wird die Resistenz nur durch zwei Mutationen innerhalb der für die erste Transmembrandomäne kodierenden Region des *Lr34sus*-Allels hervorgerufen, die eine Phenylalanindeletion und einen Austausch von Tyrosin zu Histidin bewirken. Reis-, Hirse-, Mais- oder Gerstenpflanzen können durch eine Transformation mit dem *Lr34res*-Gen sogar gegen ihre jeweiligen Krankheitserreger, zu denen einige spezialisierte Mehltau und Rostarten zählen, resistent gemacht werden. Jedoch kann eine starke Expression des Transgens auch gewisse Nebeneffekte, wie z.B. eine Induktion von Trockenstress in Reis (S. Krattinger, persönliche Mitteilung), eine Akkumulation von Triglyceriden im Gerstenblattgewebe oder eine Ausbildung von Blattspitzennekrosen als Anzeichen verfrühter Seneszenz, bewirken. Die Zielsetzung dieser Arbeit bestand in der Untersuchung des molekularen Mechanismus des ABC-Transporters, der eine Erklärung für die Schädlingsresistenz liefert und diese in einen Zusammenhang mit den übrigen beobachteten Nebeneffekten bringt.

Als erstes wurde die subzelluläre Lokalisierung des ABC-Transporters aufgeklärt. Aufgrund der Abwesenheit von Signalpeptiden und der Studien zu verwandten PDR-Transportern wurde vermutet, dass Lr34 in die Plasmamembran (PM) eingebaut wird. Und tatsächlich konnte durch eine Auftrennung der Gesamtmembranfraktion von *Lr34res*-exprimierenden BY2-Zellen in subzelluläre Membranfraktionen mittels Dichtezentrifugation eine starke Korrelation des HA-getaggten Proteins mit dem Plasmamembranmarker nachgewiesen werden. Dieses Ergebnis war die Grundlage für nachfolgende Untersuchungen zur Funktionalität und zur Aufklärung des Substratspektrums des ABC-Transporters. Einige Indizien, die auf transkriptomischen Veränderungen in den jeweiligen transgenen Pflanzen beruhen, führten zu der Vermutung, dass Lr34res eine Rolle im Lipidmetabolismus spielt. Denn zum einen spielen bestimmte Lipide eine herausragende Rolle als Signalmoleküle bei biotischen und abiotischen Stressreaktionen zur Aktivierung der entsprechenden Signaltransduktionskaskaden, und zum anderen wurde in zahlreichen Studien eine direkte Rolle von ABC-Transportern – insbesondere aus der G-Unterfamilie – bei der Translokation polarer Lipide innerhalb der Membran nachgewiesen, während weitere Studien Störungen der Fetteinlagerungen beim Menschen auf eine Fehlfunktion bestimmter ABC-Transporter zurückführen konnten. Schließlich lieferten auch die transkriptomischen Daten von *Lr34res*-exprimierenden Reis- (S.

Krattinger, persönliche Mitteilung) und Gerstenpflanzen Hinweise auf eine direkte Beeinflussung des zellulären Lipidmetabolismus, was in Gerste beispielsweise zu einer Akkumulation von Triglyceriden führt. Folglich wurde als nächstes untersucht, ob auch Lr34res dazu in der Lage ist, bestimmte Phospholipide (PL) von einer Membraneinzelschicht auf die andere zu übertragen, was als PL flipping (exoplasmatisch nach cytoplasmatisch) oder PL flopping (cytoplasmatisch nach exoplasmatisch) bezeichnet wird. Dazu wurden in einem ersten Versuch *Lr34res*-exprimierende Gerstenprotoplasten mit fluoreszenzmarkierten PLen inkubiert, um deren zeitabhängige Aufnahme zu quantifizieren. Mit diesem Experiment konnte gezeigt werden, dass Phosphatidat (PA) in den transgenen Protoplasten stärker akkumuliert, während Phosphatidylserin (PS) verstärkt exportiert wird. Bei der Aufnahme von Phosphatidylglycerin (PG), Phosphatidylethanolamin (PE) und Phosphatidylcholin (PC) konnten hingegen keine Unterschiede zwischen transgenen und Kontroll-Protoplasten festgestellt werden. Um diese Ergebnisse zu bestätigen, wurden in einem weiteren Experiment Tabak BY2 Protoplasten mit PL-Biosensor-Konstrukten zur Visualisierung der subzellulären Verteilung bestimmter PLe in Abhängigkeit von Lr34res transformiert. Zusammenfassend konnte mit dieser Methode in Übereinstimmung mit den vorherigen Resultaten eine Reduktion des PS-Gehalts auf der cytoplasmatischen Seite der PM von Lr34res-exprimierenden Protoplasten gezeigt werden. Interessanterweise wurde auf dieser Membranseite zusätzlich eine Reduktion des Phosphatidylinositol-(4,5)-bisphosphat-Gehalts durch die Aktivität von Lr34res beobachtet. Als letztes wurde der ABC-Transporter heterolog in *Saccharomyces cerevisiae* exprimiert, um Wachstumsexperimente mit Toxinen, durch die eine Exposition bestimmter PLe in der PM bzw. ein verstärkter PL-Import nachgewiesen werden kann, durchzuführen. Auch in diesem Fall wurde eine Exposition von PS in der PM beobachtet, da die Sensitivität für das PS-spezifische Toxin Papuamid A in *Lr34res*-exprimierenden Hefezellen erhöht war. Allerdings konnte durch die Expression von *Lr34sus* derselbe Effekt erzielt werden, was darauf hindeutet, dass beide Transportervarianten als PS-Floppasen fungieren. Dagegen deuteten die Wachstumstests mit dem PE-spezifischen Toxin Duramycin auf eine reduzierte Exposition von PE in der PM – insbesondere durch die Expression von Lr34res – hin. Folglich wird zumindest in Hefen nicht nur PS, sondern vermutlich auch PE als Substrat von Lr34res und Lr34sus erkannt und von der PM-Innenseite auf die PM-Außenseite bzw. umgekehrt übertragen. Zusammenfassend konnte in allen Systemen übereinstimmend gezeigt werden, dass Lr34res (und Lr34sus) über eine PS-Floppase-Aktivität verfügen und eventuell weitere PLe als Substrat erkennen können.

Die Expression von Lr34res in einer temperatursensitiven Hefemutante mit einem Defekt in einem essentiellen PL transferprotein führte zu einer Akkumulation von neutralen Lipiden in sogenannten Ölkörpern, gekennzeichnet durch eine Vergrößerung des Organellvolumens und deren Anzahl.

Interessanterweise wurde dieser Effekt trotz ähnlicher Expressionslevel nur für Lr34res und nicht für Lr34sus beobachtet, was auf unterschiedliche Funktionen der beiden Transporter hindeutet. Da eine solche Ölakkumulation bereits in *Lr34res*-exprimierenden Gerstenblättern nachgewiesen wurde, liegt die Vermutung nahe, dass die Aktivität von Lr34res tatsächlich einen Einfluss auf den gesamten zellulären Lipidmetabolismus hat.

Da physiologische Stressreaktionen sehr oft mit der Induktion von Enzymen zur Umwandlung von membrangebundenen PLen verbunden sind, wurde vermutet, dass Lr34res deren Aktivität möglicherweise indirekt beeinflusst. Allerdings wurden zumindest in transgenen Gerstenpflanzen keine Indizien für einen verstärkten oder verminderten Abbau der häufigsten Membranlipide gefunden. Daher ist eher zu vermuten, dass Lr34res entweder indirekt die Bildung von schwach konzentrierten Signallipiden (Polyphosphoinositiden) stimuliert oder dass die Stressreaktionen direkt durch die PL-Translokationsaktivität von Lr34res verursacht werden.

In einem weiteren Experiment wurde der ABC-Transporter heterolog in Hefen (*Saccharomyces cerevisiae*) und In Tabak (*Nicotiana tabacum*) BY2 Zellen exprimiert, um große Proteinmengen für eine nachfolgende Aufreinigung und Rekonstitution in Proteoliposomen zu produzieren. Denn eine Analyse des genauen Substratspektrums sowie der Aktivitätsunterschiede zwischen Lr34res und Lr34sus können nur durch PL-flipping-Experimente mit aufgereinigtem Lr34-Protein, das in Liposomen mit definierter Lipidzusammensetzung rekonstituiert wurde, durchgeführt werden. Ein Vergleich der beiden Expressionssysteme zeigte, dass BY2 Zellen einen höheren Proteinertrag lieferten als Hefezellen, und dass das Lr34-Protein effizient aus der pflanzlichen Membranfraktion solubilisiert werden konnte. Aber obwohl der HA-getaggte ABC-Transporter erfolgreich aus der solubilisierten BY2 Membranfraktion aufgereinigt werden konnte, war selbst die aus Großkulturen erhaltene Gesamtproteinmenge bei weitem nicht ausreichend für eine anschließende Rekonstitution in Liposomen.

Interessanterweise bewirkt die Expression des ABC-Transporters in transgenen Reispflanzen eine Induktion von Trockenstress (S. Krattinger, persönliche Mitteilung). Daher wurde untersucht, ob die Hochregulierung von Trockenstressgenen tatsächlich mit einer reduzierten Transpirationsrate in *Lr34res*-exprimierenden Reisblättern einhergeht. Und tatsächlich konnte in Gasaustauschmessungen nachgewiesen werden, dass nicht nur die Transpiration, sondern auch die Photosyntheseaktivität infolge einer eingeschränkten Leitfähigkeit der Spaltöffnungen vor allem bei starker Transgenexpression reduziert sind.

Zusammenfassend wurde festgestellt, dass der ABC-Transporter Lr34 die Verteilung bestimmter PLe innerhalb der Membran verändert, was Konsequenzen für das membranassoziierte Proteom hat. Genauer gesagt werden PS (und Phosphatidylinositol-(4,5)-bisphosphat)-bindende Proteine vermutlich von der Membran losgelöst und diffundieren daraufhin ins Cytosol, während PA-bindende Proteine verstärkt zur Membran rekrutiert würden. Diese Veränderungen könnten daraufhin bestimmte Signaltransduktionskaskaden aktivieren, die zu den beobachteten Phänotypen in *Lr34res*-exprimierenden Pflanzen, wie z.B. erhöhter Schädlingsresistenz, Trockenstressinduktion oder Ölakkumulation, führen würden.

List of abbreviations

ABA	Abscisic acid	PC	Phosphatidylcholine
ABC	ATP-binding cassette	PCD	Programmed cell death
ADP	Adenosine diphosphate	PDK	Phosphoinositide-dependent kinase
ATP	Adenosine triphosphate	PDR	Pleiotropic drug resistance
BY2	Bright yellow 2	PE	Phosphatidylethanolamine
DAG	Diacylglycerol	PG	Phosphatidylglycerol
DAMP	Damage-associated molecular pattern	PIP	phosphatidylinositolphosphate
DDM	n-Dodecyl β-D-maltoside	PI3K	Phosphoinositide 3-kinase
DGDG	Digalactosyldiacylglycerol	PKC	Protein kinase C
DGPP	Diacylglycerolpyrophosphate	PL	Phospholipid
ER	Endoplasmic reticulum	PLA	Phospholipase A
ETS	Effector-triggered susceptibility	PLase	Phospholipase
FA	Fatty acid	PLC	Phospholipase C
FAD	Flavin adenine dinucleotide	PLD	Phospholipase D
GPCR	G protein-coupled receptor	PM	Plasma membrane
HA	Human influence hemagglutinin	PR	Pathogenicity-related
HRP	Horse radish peroxidase	PRR	Pattern recognition receptor
IP_3	Inositol trisphosphate	PS	Phosphatidylserine
JA	Jasmonic acid	RFP	Red fluorescent protein
LCB	Long-chain base	RLK	Receptor-like kinase
LRR	Leucine-rich repeat	ROS	Reactive oxygen species
MAMP	Microbe-associated molecular pattern	SA	Salicylic acid
MGDG	Monogalactosyldiacylglycerol	SDS	Sodium dodecyl sulfate
NAD	Nicotinamide adenine dinucleotide	TAG	Triacylglycerol
NBD	7-nitro-2-1,3-benzoxadiazol-4-yl (PLs)/ nucleotide-binding domain	TCA	Tricarboxylic acid
PA	Phosphatidic acid	TMD	Transmembrane domain
PAK	Phosphatidic acid kinase	VLCFA	Very long-chain fatty acid
PAMP	Pathogen-associated molecular pattern		

1 General Introduction

1.1 Lipid biosynthesis in plants

In eukaryotic cells glycerolipid biosynthesis is highly compartmentalized. Starting with the conversion of carbohydrates into precursor molecules such as glycerol-3-phosphate during glycolysis in the cytoplasm and citrate for the generation of acetyl-CoA during the tricarboxylic acid (TCA) cycle in mitochondria, these substrates can be used in the endoplasmic reticulum (ER) for the biosynthesis of phospholipids (PLs) and neutral glycerolipids (Ohlrogge *et al*., 1995). The first important intermediate of glycerolipid biosynthesis is phosphatidic acid (PA), which is at the same time the decisive factor for the interconversion of PLs and neutral lipids. While sterol and PL biosynthesis completely take place in the ER, complex sphingolipids are produced from ER-derived ceramide in the Golgi apparatus (Gault *et al.*, 2010). In addition, PA can be exported to mitochondria for the biosynthesis of cardiolipin (Gohil *et al*., 2004).

The situation in plants is even more complex because plastids take part in lipid production (Fig. 1.1; Mekhedov *et al*., 2000). For example, the most prominent glycolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are exclusively synthesized in these organelles and therefore constitute the major components of plastidic membranes (Kobayashi *et al*., 2009). Moreover, plastids are an important source of fatty acids (FAs), especially for the monounsaturated oleic acid (18:1) and further polyunsaturated FAs, such as linoleic acid (18:2) and linolenic acid (18:3). In their CoA-esterified form these intermediates are exported from plastids and then incorporated into glycerolipids in the ER. As animals are not able to synthesize these unsaturated FA classes by themselves, they must take them up with their nutrition in order to maintain a functional lipid metabolism and prevent diseases associated with a deficiency in essential FAs (Calon & Cole, 2007).

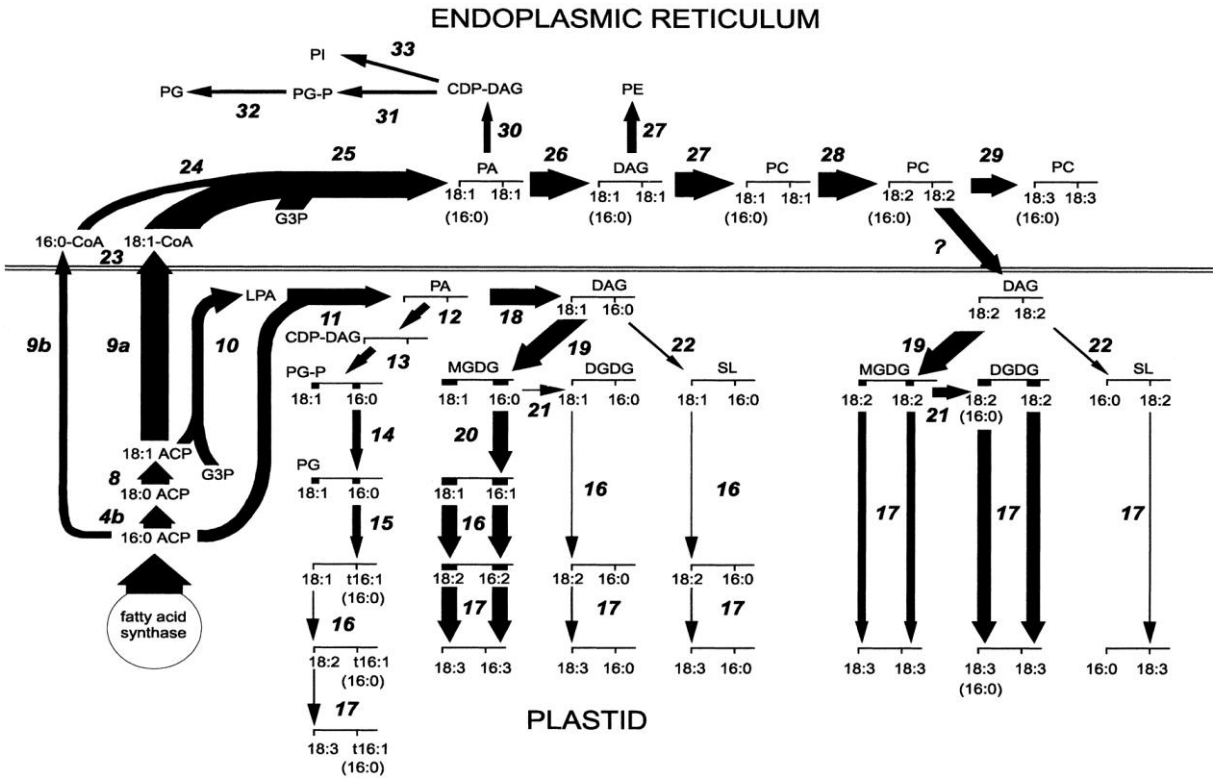

Figure 1.1 Biosynthetic pathway of membrane lipids in *Arabidopsis thaliana* | Glycerolipid biosynthesis occurs in different cellular compartments in plants. Plastids are the sites of galactolipid biosynthesis and provide a large FA-CoA pool for the generation of PLs in the ER. A constant exchange of lipid intermediates between both compartments allows an efficient adjustment of the PL/galactolipid ratio. Widths of the arrows show the relative fluxes through different reactions. Numbers represent enzymes catalyzing the corresponding reactions (not mentioned here). Abbreviations: G3P, glycerol-3-phosphate; LPA, 1-acyl-glycerol-3-phosphate; PA, phosphatidic acid; DAG, dyacylglycerol; CDP-DAG, cytidine-5'-diphosphate-diacylglycerol; PG, phosphatidylglycerol; PG-P, phosphatidylglycerol-3-phosphate; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SL, sulfoguinovosyldiacylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine. (source: Mekhedov, S., de Ilárduya, O. M., & Ohlrogge, J. (2000). Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis. Plant Physiology, 122(2), 389-402)

Depending on the environmental conditions and the developmental stage lipid biosynthesis can be stimulated. For example, the unicellular green alga *Chlamydomonas reinhardtii* produces large amounts of neutral lipids under nitrogen-deprivation, which results in the formation of intracellular oil bodies (Wang *et al*., 2009). These organelles are derived from microdomains of the ER where lipid biosynthetic enzymes assemble and fill the interbilayer space of the membrane with newly synthesized neutral lipids (Kohlwein *et al*., 2013). These thickened membrane structures can be released from the ER facilitated by associated proteins in three possible ways (Fig. 1.2A-C) and accumulate in the cytosol as oil bodies, shielded from the aqueous cytosolic lumen by a PL monolayer. As lipid reservoirs oil bodies play crucial functions for the biosynthesis of membrane

lipids from triacylglycerols (TAGs). On the other hand, accelerated membrane degradation promotes the formation of neutral lipids, which are stored in oil bodies and consequently lead to their enlargement. This ability reflects the importance of these organelles in balancing lipid biosynthesis and degradation.

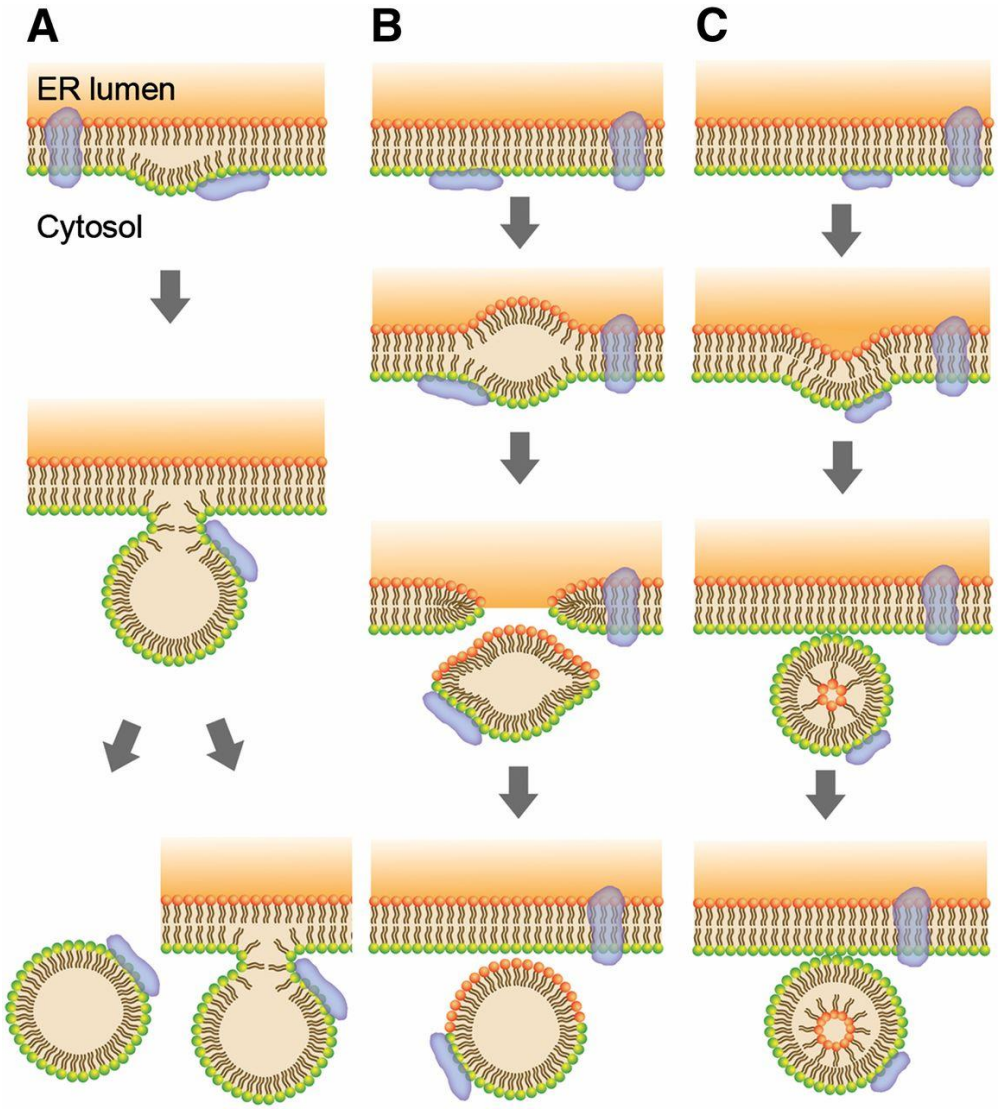

Figure 1.2 Models of oil body formation | (a) In the "lensing model" neutral lipids are deposited between the two ER monolayers so that the neutral lipid core bulges out on the cytosolic side after having reached a critical size and may get released from the ER or stay attached to it. (b) The bicelle formation also requires the deposition of neutral lipids between the ER monolayers but in this case oil bodies are excised from the ER with the aid of specific proteins. (c) The neutral lipid core of oil bodies could alternatively by formed by a rearrangement of the inner ER leaflet so that the process of oil body biogenesis rather resembles vesicle formation. The different colors indicate the origin of the PL monolayer. Blue structures represent proteins involved in oil body formation. (source: Kohlwein, S. D., Veenhuis, M., & van der Klei, I. J. (2013). Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat—store'em up or burn'em down. Genetics, 193(1), 1-50.)

1.2 Lipid catabolism – β-oxidation of fatty acids

Storage lipids constitute an important source of energy, especially for the maintenance of metabolism during starvation or extensive growth phases. First, they have to be degraded in order to convert the stored energy into a form that can be used by the organism for the biosynthesis of ATP. The initial step requires the release of free fatty acids (FAs) from storage lipids catalyzed by lipases and their consecutive activation by esterification to CoA (Zechner *et al.*, 2005). Afterwards, the activated FAs are imported into mitochondria by specific transport proteins for β-oxidation. This catabolic pathway includes an initial dehydrogenation step for the generation of 2-trans-enoyl CoA, which is then converted to L-3-hydroxy acyl CoA (Fig. 1.3). Further dehydrogenation yields 3-ketoacyl CoA that is finally split by thiolysis into acetyl-CoA and a shortened FA for the reinitiation of the β-oxidation cycle. In the end, this process generates large amounts of $FADH_2$ and $NADH+H^+$ as reducing power for the generation of ATP in the mitochondrial oxidative phosphorylation. The released acetyl-CoA is oxidized to CO_2 in the citric acid cycle.

While saturated FAs can easily pass β-oxidation, unsaturated FAs must be processed by isomerases (and reductases for even-numbered double bonds) in order to be recognized as substrate for acyl-CoA dehydrogenase (Kunau & Dommes, 1978). Moreover, very long chain FAs (>22 C-atoms) or very complex FAs have to undergo initial oxidation steps in peroxisomes before they can enter mitochondrial oxidation starting from octanoyl-CoA (Singh *et al*., 1987). Interestingly, it was found that the import of very long chain FAs into peroxisomes requires an ABC transporter of the D-subfamily as defects in the corresponding gene for example lead to perturbations in lipid mobilization associated with reduced fertility in the *Arabidopsis thaliana comatose* (ABCD1) mutant (Footitt *et al*., 2007).

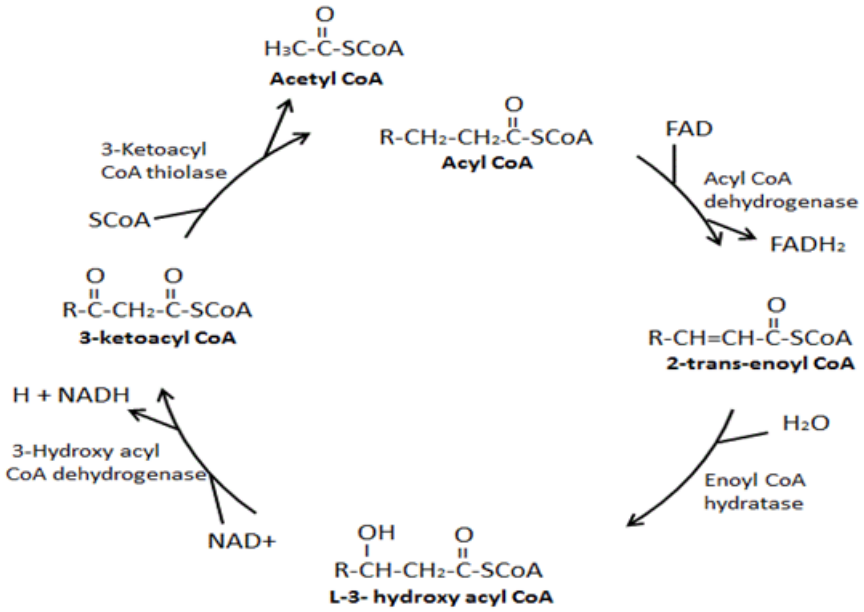

Figure 1.3 Major reactions of mitochondrial β-oxidation of FAs | The CoA-esterified form of FAs is converted to acetyl-CoA in four consecutive enzymatic steps that involve dehydrogenation, hydration, oxidation and thiolysis. This process is repeated until the entire FA is degraded. Abbreviations: FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide. (source: http://lipidlibrary.aocs.org)

1.3 Features of biological membranes

Each cell is surrounded by a membrane, a lipid bilayer, which is separating the cell from its environment. In eukaryotes, membranes are furthermore essential to divide the cell into various reaction compartments. As organelles they fulfill crucial functions for the survival of the organism. For example, protein synthesis starts at the ribosomes of the endoplasmic reticulum (ER), which is basically a folded membrane structure enclosing the nucleus, and continues in the Golgi apparatus, a multi-vesicular compartment, where proteins are modified and delivered to their final destination (Lodish *et al.*, 2000). Interestingly, some organelles like mitochondria or plastids even contain a double membrane layer, which is a clear sign for their prokaryotic origin and an endocytic event according to the endosymbiotic theory (Zimorski *et al*., 2014). On the basis of the lipid composition it is assumed that the outer layer is derived from the eukaryotic host cell while the inner is provided by the engulfed prokaryotic cell.

Biological membranes are composed of different lipid classes, which are phospholipids (PLs), glycolipids and sterols (Fig. 1.4; Fantini *et al*., 2002). Their structure can be very complex as the fatty acid (FA) moieties have different degrees of unsaturation (and hydroxylation in case of sphingolipids),

while the hydrophilic head group can also be modified, *e.g.* by phosphorylation. With their FA tails these polar lipids build the hydrophobic core of the membrane, while their hydrophilic head groups are exposed to the aqueous phase of the respective compartment. Membrane lipids not only interact with each other but also with proteins that either cross the lipid bilayer in case of transmembrane proteins or are anchored to the membrane by posttranslational modifications, namely myristoylations and prenylations (Resh, 2013). In addition, electrostatic interactions between specific proteins and the polar head groups of the lipids play a crucial role for their recruitment to the membrane (Do Heo *et al.*, 2006). This feature is of outstanding importance for the activation of several cellular signal transduction cascades and will be discussed in more detail in another chapter.

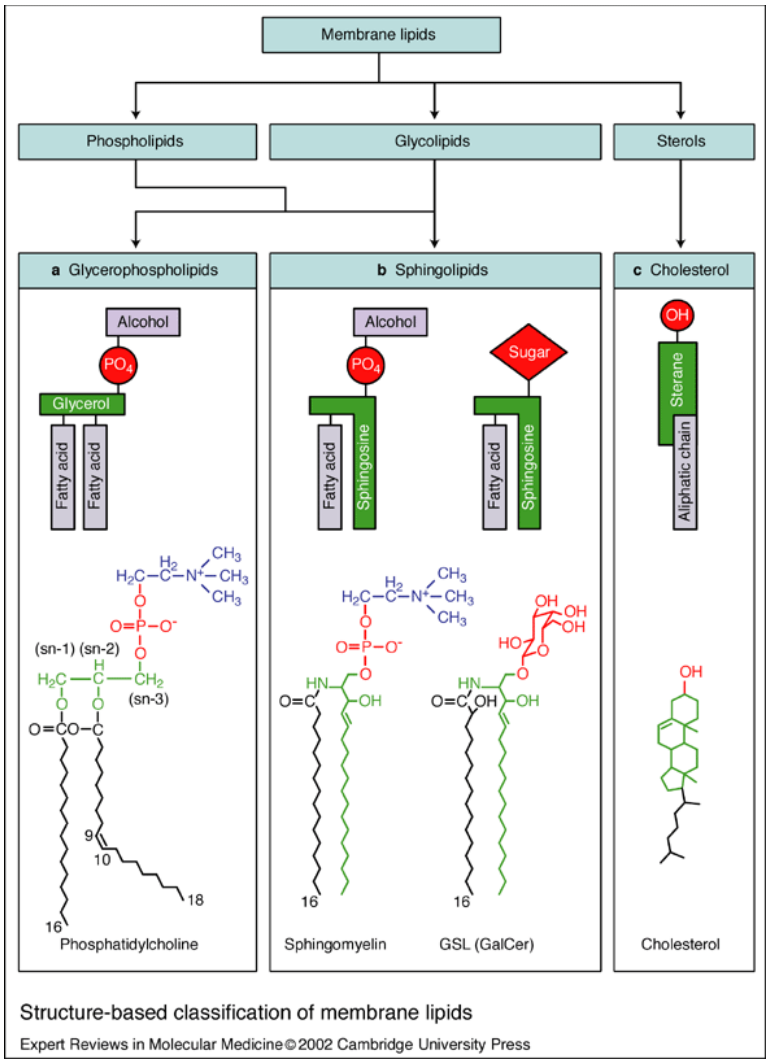

Figure 1.4 Classes of membrane lipids| Biological membranes are composed of three different major amphiphilic lipid classes, namely phospholipids, sphingolipids and cholesterol. All of them contain at least one fatty acid moiety with variable length and degree of saturation (black), a characteristic backbone (green) and a polar head group (red), which can be esterified to different alcoholic compounds, *e.g.* choline. (source: Fantini, J., Garmy, N., Mahfoud, R., & Yahi, N. (2002). Lipid rafts: structure, function and role in HIV, Alzheimer's and prion diseases. Expert reviews in molecular medicine, 4(27), 1-22)

The relative proportion of the different lipid classes significantly determines the physical properties of the membrane. For example, sterols and sphingolipids can be enriched at certain domains of the membrane and form so-called lipid rafts (Fig. 1.5; Van Anthony *et al*., 2016). These domains are characterized by a greater thickness and density compared to the surrounding areas and play important roles *i.a.* in protein trafficking or the assembly of G protein complexes for signal transduction. Furthermore, these physical properties are the reason why proteins embedded in lipid rafts cannot be extracted with detergents. The density of the lipid bilayer additionally affects the permeability for solutes and gases, which is especially relevant for the diffusion of oxygen under hypoxic conditions favoring a more gas-permeable, less dense (poor in cholesterol) membrane structure (Subczynski *et al*., 1991).

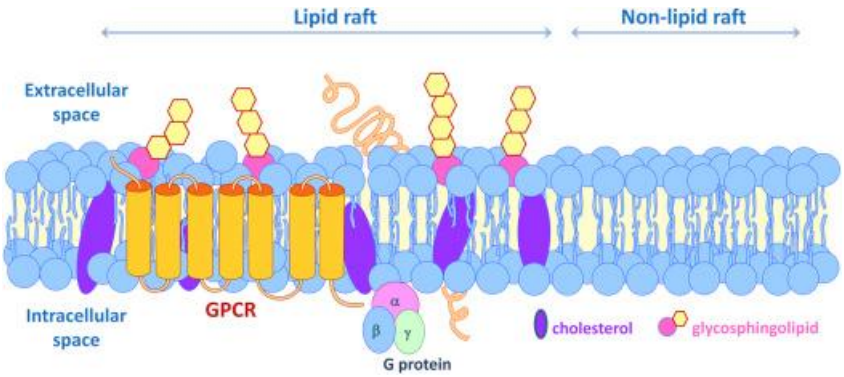

Figure 1.5 Structure of lipid rafts | Biological membranes mostly consist of a phospholipid bilayer, in which membrane proteins are embedded. However, certain domains of the membrane are enriched in other lipid classes such as sterols and glycosphingolipids with impact not only on the physical properties of these membrane sites but also on the associated proteome. These so-called lipid rafts fulfill crucial functions in cellular signaling, endocytotic and exocytotic events.For example, G protein-coupled receptors (GPCRs) are mainly found at these sites of the membrane. (source: Van Anthony, M. V., Cuevas, S., Zheng, X., & Jose, P. A. (2016). Localization and signaling of GPCRs in lipid rafts. Methods in cell biology, 132, 3-23)

PLs constitute the most abundant component of biological membranes. They can be distinguished by their hydrophilic head group and by the length and degree of unsaturation of their fatty acid tails, which significantly affects the fluidity of the membrane (Kates *et al.*, 1984). The most prominent PLs are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI), which can be further phosphorylated to phosphatidylinositolpolyphosphates (PIPs) (Fig. 1.6). Their relative ratio may differ from one organism to another and even within the membrane bilayer their distribution is not even, as PE and PS are usually enriched in the cytoplasmic leaflet, while PC is mainly present in the exoplasmic leaflet (Zachowski *et al*., 1993). This asymmetry is generated along the secretory pathway by so-called PL flippases and floppases that move certain PLs from the exoplasmic to the cytoplasmic membrane

leaflet and the other way round, respectively. These transporter classes will be described in more detail in a separate chapter. An interbilayer translocation of PLs is for instance crucial for the generation of vesicles in the Golgi apparatus or for endo- and exocytic processes (Gall *et al*., 2002; Hua *et al*., 2002). The transport of PLs from their origin in the ER to their final cellular destination either happens via vesiculation or is facilitated by PL transfer proteins, whose precise role will also be discussed in a separate chapter.

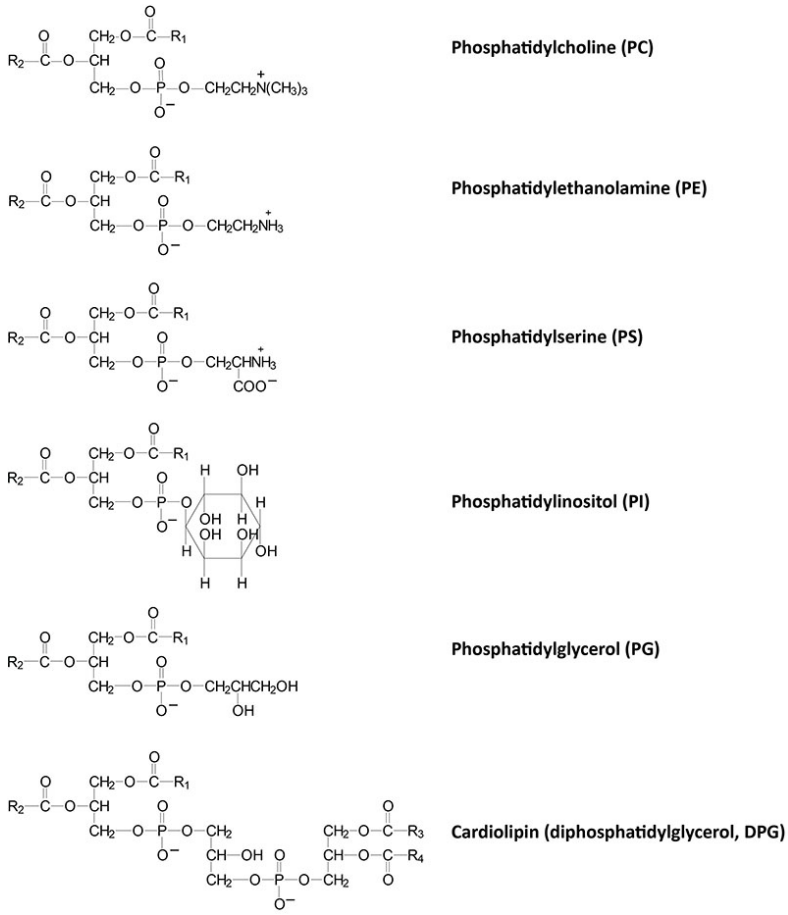

Figure 1.6 Major phospholipid species | In every organism phosphatidylcholine (PC) and phosphatidylethanolamine (PE) constitute the majority of membrane PLs. As their alcoholic head groups contain a positive charge they are overall neutral, whereas phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) are negatively charged. With their different sterical and electrical properties they significantly determine the physical properties of biological membranes (source: https://themedicalbiochemistrypage.org/lipid-synthesis.php)

Biological membranes are subject to constant modifications and rearrangements, especially when environmental conditions change. For example, membrane fluidity at low temperatures can only be

guaranteed by a high degree of unsaturation of the fatty acid moieties, catalyzed by lipid desaturases (Kodama *et al*., 1994). These adaptations are crucial for maintaining the functionality of intrinsic membrane proteins. External factors can also stimulate the modification of PLs by the induction of phospholipases (PLases) (Spiegel *et al*., 1996; Munnik *et al*., 1998; Munnik, 2001; Wang *et al*., 2002; Wang, 2005; Xue *et al*., 2007; Zhao, 2015). These enzymes convert structural lipids into signaling molecules for the initiation of signal transduction cascades. The precise role and mode of function of PLases will be discussed in a separate chapter. In general, biological membranes must not be considered as fixed cell borders comparable to the cell wall but as dynamic structures, which are under constant modification by lipid delivery via the secretory pathway and lipid detraction by endocytic events. In the end, all these processes guarantee rapid and highly flexible adaptations of the membrane lipid profile to changing environmental conditions.

1.4 Lipid transport and distribution

From their site of synthesis in the ER, membrane lipids have to be delivered to their final cellular destinations. This happens to a major part via the secretory pathway, in which vesicles consisting of proteins and polar lipids are released from the ER to enter the Golgi apparatus for further modifications and the consecutive sorting to their target sites (Van Meer *et al.*, 2008). By this mechanism the secretory pathway creates a route for lipid trafficking from their origin in the ER to their destinations in the Golgi apparatus, the PM or endosomal compartments. However, this process requires the formation of vesicles at the site of origin and their fusion with the target membrane, which is facilitated by flippase-mediated redistribution of polar lipids from the luminal to the cytoplasmic membrane side and the formation of a protein coat on the vesicle surface (Van Meer et al., 2008; Graham, 2004). Another way of lipid exchange between two distant membranes is a transfer by specialized lipid transfer proteins that can extract certain lipids from one membrane and transfer them through the cytoplasm to another (Fig. 1.7; Lev, 2010). A defect in lipid transfer protein function leads to aberrant lipid distribution, which typically goes along with a temperature sensitive phenotype, as for instance observed in *S. cerevisiae* mutants defective in the Sec14 PL transfer protein (Curwin *et al*., 2009).

Lipid transfer not only happens between two different membranes but also within a membrane. As already mentioned before, the distribution of lipids between the two monolayers is usually not symmetrical (except of the ER) because PE and PS are mainly enriched in the cytoplasmic leaflet,

while PC and sphingolipids are mainly present in the exoplasmic membrane leaflet. This asymmetry is generated along the secretory pathway by energy-dependent PL flippases (exoplasmic to cytoplasmic) and floppases (cytoplasmic to exoplasmic). For example, P_4-ATPases have been characterized as PL flippases responsible for maintaining high PE and PS levels in the cytoplasmic PM leaflet (Lopéz-Marqués *et al*., 2010; Coleman *et al*., 2009; Alder-Baerens *et al*., 2006). In most cases they must form a complex with an additional CDC50-family protein as β-subunit to guarantee full activity and proper localization (Poulsen *et al*., 2008; Lopéz-Marqués *et al*., 2012). Apart from increasing the temperature sensitivity, defects in P_4-ATPases can for example lead to impaired pollen fitness or root growth in *A. thaliana* (Poulsen *et al*., 2008; McDowell *et al*., 2013). On the other hand, ABC transporters have been shown to be involved in the reverse transfer of lipids from the cytoplasmic to the exoplasmic PM leaflet. Several members of the A-, C- and G-subfamilies of mammalian ABC transporters are responsible for the exposure of diverse lipids, such as the PLs PC, PE and PS, the sterols cholesterol and phytosterol or sphingosine-1-P (Borst *et al*., 2000; Sabeva *et al*., 2009). Instead of recruiting a β-subunit, half-size ABC transporters need to form homo- or heterodimers to be active, while full-size transporters, *e.g.* the members of the pleiotropic drug resistance (PDR) class, already constitute functionally active units. Examples of ABC transporters involved in lipid metabolism and their physiological functions will be discussed in detail in the next section. The third class of PL translocators are scramblases that – in contrast to P_4-ATPases and ABC transporters – are not energy-dependent. The activation of scramblases by an increasing cytosolic Ca^{2+} concentration promotes the breakdown of PL asymmetry in the PM with severe consequences for the cellular fate (Fadeel & Xue, 2009). Because especially the exposure of PS is a universal trigger for apoptosis and – at least in animals – in addition serves as an "eat-me" signal for macrophages (Segawa *et al*., 2014).

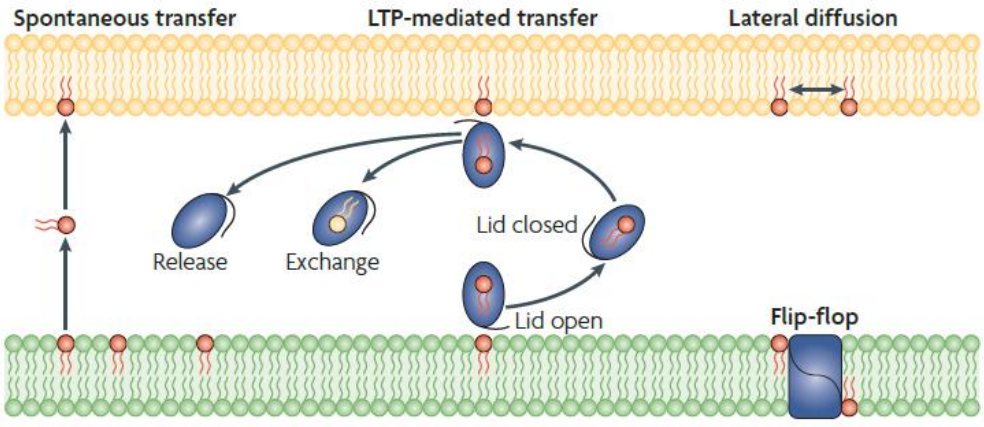

Figure 1.7 Non-vesicular lipid transport| Lipids can move by lateral diffusion within a membrane monolayer, mainly restricted by the membrane stiffness. Because of their hydrophilic head group, most PLs can hardly cross the hydrophic core of the membrane and therefore have to be actively "flipped" to the opposite membrane side by specialized proteins.

The exchange of lipids between two membranes can either occur spontaneously if the membranes are in close proximity or is facilitated by lipid transfer proteins, especially when long-distance transport is required. (source: Lev, S. (2010). Non-vesicular lipid transport by lipid-transfer proteins and beyond. Nature reviews Molecular cell biology, 11(10), 739-750)

1.5 ABC transporters

Even if membranes isolate the cell from the surrounding medium or organellar lumen from the cytosol, an efficient exchange of metabolites between all cellular compartments has to be guaranteed. Therefore, proteins are embedded in the membrane that work as selective solute channels or transporters. ATP-binding cassette (ABC) transporters belong to the class of primary active transporters as they use the energy provided by the hydrolysis of ATP for the movement of various compounds against their concentration gradient (Higgins, 2001). They exist either as full-size or as half-size transporters that homo- or heterodimerize with other half-size ABC transporters. An active unit always consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) containing Walker-A and Walker-B motifs for the hydrolysis of ATP (Higgins, 1992). ABC transporters can be divided into four different classes according to their structural features, which are B-family ABC exporters, type 1 ABC importers, type 2 ABC importers and ECF-type ABC importers (Fig. 1.8A; Locher, 2016). Type 1 ABC importers mediate the uptake of diverse nutrients including ions, sugars, amino acids, short peptides and oligosaccharides in bacteria with the maltose transporter from *E. coli* being the most prominent example (Chen *et al*., 2001). The characteristic feature of this transporter class are conformational changes of the TMDs with alternating access to the substrate-binding pocket driven by the hydrolysis of ATP that mediate the import of these substrate into the cell (Fig. 1.8B). In contrast, type 2 ABC importers create peristaltic forces within their TMD for substrate release into the cytoplasm. During this process the transporter conformation passes through an asymmetrical state, which is a unique feature of type 2 ABC importers. Typically, their substrates are more hydrophobic and include *e.g.* cobalamine or iron-heme complexes (Borths *et al*., 2005; Braun *et al*., 2011; Köster, 2001). A prominent example of this ABC transporter class is the *E. coli* vitamin B12 transporter BtuCD-F. Although ECF-type ABC transporters as important vitamin importers in bacteria fulfill similar physiological functions, their structure is significantly different from type 1 and type 2 transporters because they lack periplasmic substrate-binding proteins required for substrate delivery (shown in Fig. 1.8 in red). The well-studied folate importer EcfAST from *Lactobacillus brevis* is one member of ECF-type ABC transporters. However, most ABC transporters belong to the B-family that is further divided into the subfamilies A-G and has members in almost every organism including eukaryotes. Two different mechanisms of substrate export have

been proposed for this transporter class: In the alternating access model the substrate gets access to the binding pocket through the inward-open TMD and is then released to the exoplasmic side by conformational changes of the TMD in the outward-open state (Fig. 1.8C). In contrast, the outward-only proposes a direct loading of the substrate into the outward-facing cavity and ATP-hydrolysis at the NBDs induces a compression of the TMD for substrate extrusion.

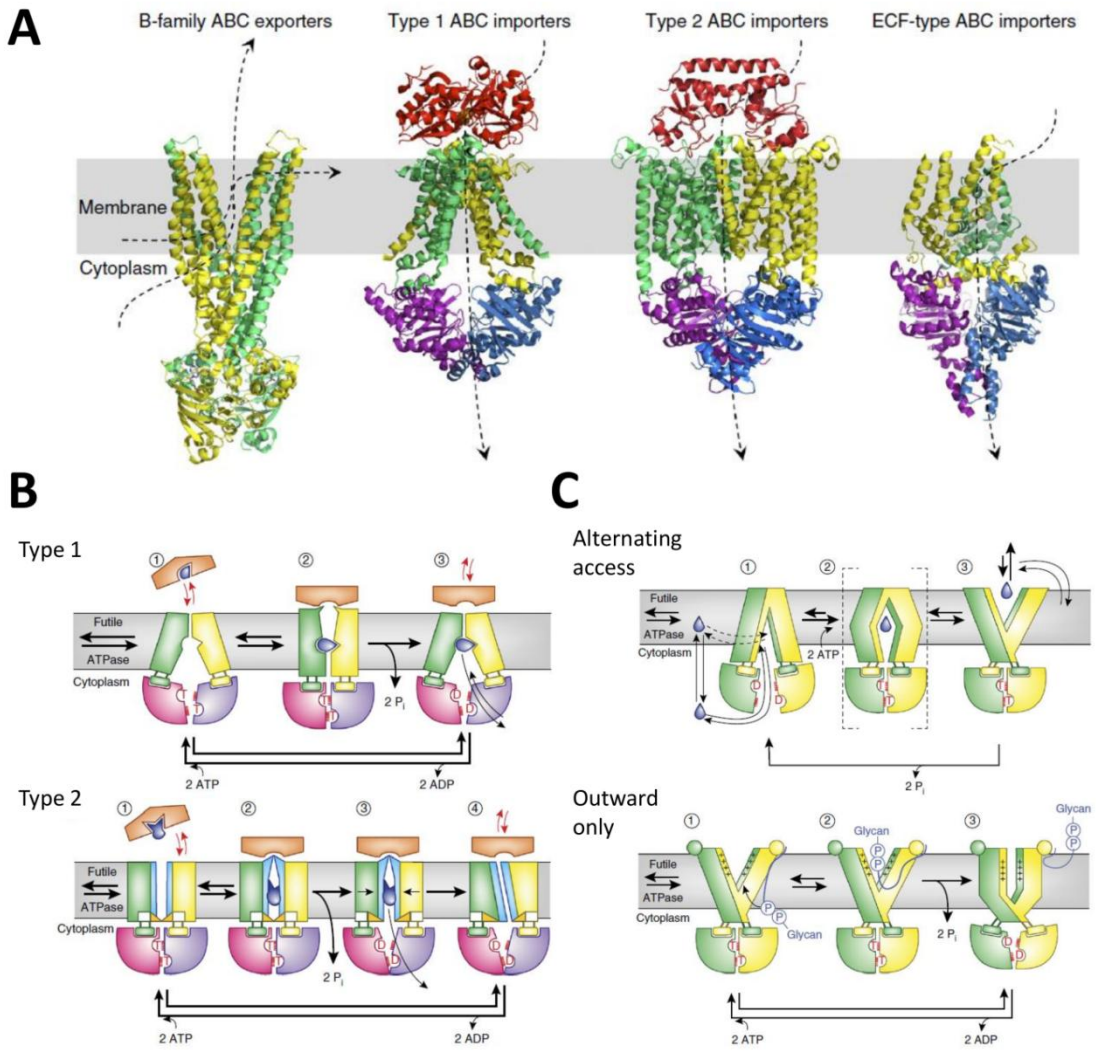

Figure 1.8 Structure-based types of ABC transporters and their proposed transport mechanism | (a) Structure of selected ABC transporters. Depicted are the models of *Staphylococcus aureus* multidrug transporter Sav1866 (B-family ABC exporters), *Archaeoglobus fulgidus* molybdate/tungstate transporter ModBC–A (type I ABC importer), *E. coli* vitamin B12 transporter BtuCD–F53 (type II ABC importer) and *Lactobacillus brevis* folate importer EcfAST87 (ECF-type ABC importer). Colors indicate: Yellow and green, TMDs; red, periplasmic substrate-binding proteins; purple and blue, NBDs in ABC importers.(b) Proposed transport mechanisms of type 1 and type 2 ABC importers. Colors indicate: Green and yellow, TMDs; pink and purple, NBDs; orange, binding proteins; dark blue, substrates; T, ATP; D, ADP. Dashed red lines in the NBDs depict the ABC signature motifs. Circled numbers denote states. See text for details. (c) Proposed alternating access and outward-only models for the transport mechanisms of B-family ABC exporters. Colors indicate: Green and yellow, half-transporters; T, ATP; D, ADP. Dotted red lines in the NBDs depict the ABC signature motifs. Circled numbers denote states.

See text for details. (source: Locher, K. P. (2016). Mechanistic diversity in ATP-binding cassette (ABC) transporters. Nature structural & molecular biology, 23(6), 487-493., modified)

While the classical role of this transporter class was seen in extrusion pumps with very broad substrate spectrum ranging from heavy metal cations to complex organic molecules (Higgins, 2001), their true physiological function goes far beyond. For instance, in plants several ABC transporters have been shown to be required for efficient hormone signaling because they can directly transport hormones from source to sink tissues. Prominent examples are the abscisic acid (ABA) exporting half-size transporter ABCG25 from *A. thaliana*, which loads ABA into the xylem for its translocation to leaves (Kuromori *et al*., 2010). At the destination tissue the phytohormone is either perceived by PM receptors or can actively be taken up into the cytosol by ABCG40, where it binds to intracellular receptors (Kang *et al*., 2010). Beside their function in hormone translocation, some ABC transporters are required for an efficient supply of wax precursors for extracellular cuticle assembly. For example, a defect in CER5 leads to altered cuticle morphology, which is assumed to be a consequence of reduced cutin monomer export by this ABC transporter (Pighin *et al*., 2004). The extracellular cell wall barrier is of outstanding importance for the interaction with pathogens. One report showed that the full-size ABCG transporter PEN3/PDR8 is required for conferring non-host resistance against several powdery mildew species (Stein *et al*., 2006). But instead of assuming a role in cell wall fortification, the authors speculated that PEN3 exports toxic compounds at the penetration sites, which would affect the viability of the pathogen.

The fact that almost half of the 48 human ABC transporters are thought to facilitate the translocation of lipids and lipid-related compounds (Tarling *et al*., 2013) highlights the importance of this transporter class for an efficient lipid metabolism and explains why their malfunction often leads to the emergence of lipid-associated diseases. For example, a defect in the human ABCD1, which is required for the import of very long chain fatty acids (VLCFAs) for β-oxidation into peroxisomes, results in the cytoplasmatic accumulation of saturated VLCFAs, a typical symptom of adrenoleukodystrophy (Wiesinger *et al*., 2013). In addition, Stargardt disease, Tangier disease and Alzheimer's disease can be traced back to deficient lipid translocation by ABC transporters mainly of the A-subfamily (Molday *et al*., 2009; Bodzioch *et al*., 1999; Abuznait *et al*., 2012). Although their real physiological substrates are still often unknown, a common mechanism is proposed which involves the translocation of lipid molecules from one side of the membrane to the other. This process is called PL flipping and the corresponding transporters are dependending on the direction of lipid movement either called flippases (exoplasmic to cytoplasmic leaflet) or floppases (cytoplasmic to

exoplasmic leaflet). Further prominent examples are the pleiotropic drug resistance (PDR)-type transporter PDR5 from *S. cerevisiae*, a PE floppase, or CDR1-3 from *Candida albicans* that have been characterized as PL translocators with distinct PL preferences and transport directions (Decottignies *et al*., 1998; Krishamurthy *et al*., 2002). In humans, an enhanced activity of the breast cancer resistance protein (BCRP/ABCG2) is for example responsible for PS exposure leading to the activation of apoptosis (Woehlecke *et al*., 2003). Although no ABC transporter from plants has so far been characterized as a PL flippase or floppase, it is highly likely that several members possess such activities *in planta*.

1.6 The resistance gene *Lr34*

The gene *Lr34res* from wheat (*Triticum aestivum*) confers partial, durable and broad-spectrum resistance against various biotrophic fungal pathogens such as powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) or stripe rust (*Puccinia striiformis*) and has been heavily used in crop breeding to reduce yield losses (Krattinger *et al*., 2009). *Lr34res* is located on the D-genome and constitutes a mutated version of the original *Lr34sus* gene, which is more similar to the orthologous genes from *Oryza sativa* (*Os*ABCG50) and *Sorghum bicolor* (*Sb*01g016775 and *Sb*01g016770), as well as to the homologous *Lr34* gene of the B-genome (Fig. 1.9). Interestingly, *Lr34res* is the only allele that can confer pathogen resistance even though both *Lr34* variants encode similar full-size ABC transporters of the G-subfamily, also known as pleiotropic drug resistance (PDR)-type transporters. Two of the three mutations in *Lr34res* lead to a phenylalanine deletion and a tyrosine to histidine conversion in the first transmembrane domain. Presumably, these modifications either affect protein stability or the activity of the transporter with impact on pathogen invasion. A typical feature of wheat plants containing the resistance gene is the development of leaf tip necrosis in the flag leaf as a sign of premature senescence, which enables a phenotypic distinction between *Lr34res* and *non-Lr34res* containing wheat varieties (Krattinger *et al.*, 2009).

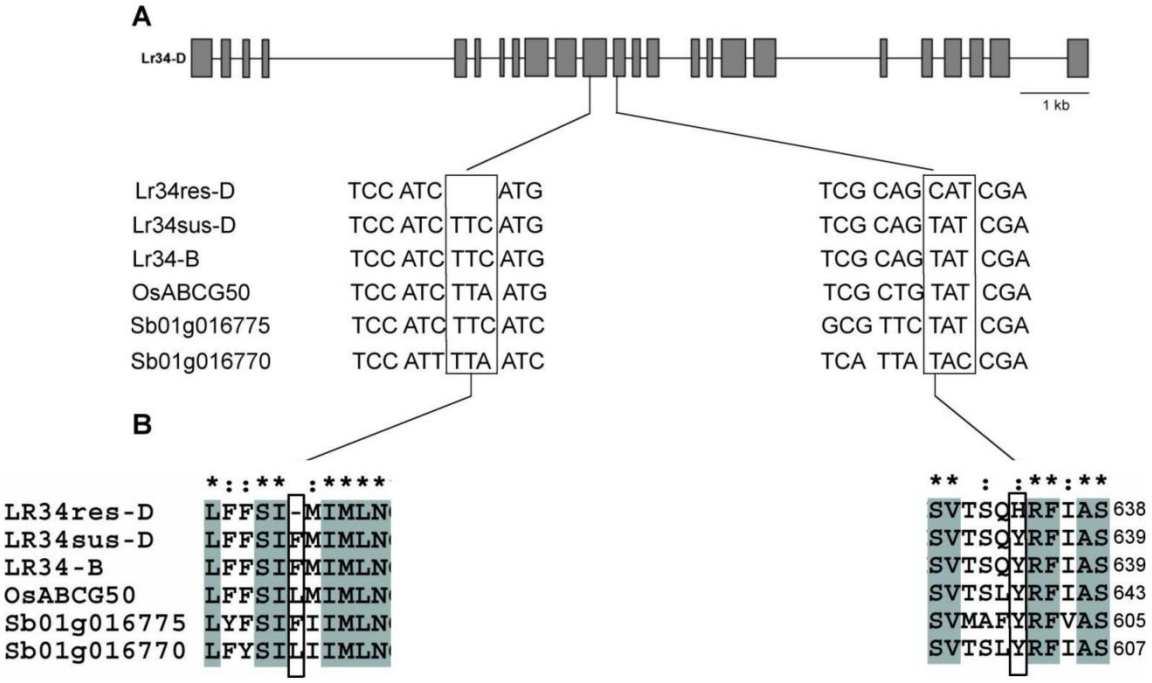

Figure 1.9 Comparison of Lr34res with homologous and orthologous genes from different cereal species | (a) *Lr34res* is located on the wheat D-genome and both crucial mutations are found in exonic sequences of the coding region for the first transmembrane domain. At the first position a whole codon triplet is deleted compared to the *Lr34sus* allele, the homologous *Lr34* gene on the wheat B-genome and the orthologous genes from *O. sativa* (*Os*ABCG50) and *S. bicolor* (*Sb*01g016775 and *Sb*01g016770). At the second position a thymine is substituted by a cytosine in *Lr34res*. (b) On the protein level the first mutation evokes a phenylalanine deletion and the second mutation a tyrosine to histidine conversion in the first transmembrane domain of the ABC transporter as compared to the *Lr34sus* allele. (figure obtained from S. Krattinger)

Recently, it was demonstrated that *Lr34res* can be functionally transferred into related crop species, such as sorghum, barley, rice or maize because all these plants gained resistance to their specific (hemi-)biotrophic pathogens by the expression of the transgene (Risk et a., 2013; Krattinger *et al*., 2016; Sucher *et al*., 2017; Schnippenkoetter *et al*., 2017). However, high expression levels of *Lr34res* can lead to an early induction of senescence, becoming visible as leaf tip necrosis. Moreover, transcriptomic analyses have revealed that several stress-responsive genes, *e.g.* pathogenicity-related (PR) genes in barley or dehydrins in rice, are upregulated in these transgenic plants in the absence of stress exposure (Chauhan *et al*., 2015; S. Krattinger, personal communication). As the accelerated aging process caused by a strong expression of the ABC transporter unfavorably goes along with reduced plant growth and total grain yield in these species, the expression level should be balanced in a way that the activity is sufficient to suppress fungal invasion, while plant fitness is not negatively affected, in order to use the resistance gene for the optimization of crop yield.

1.7 Interaction of plants with fungal pathogens

Like any other organism plants are constantly challenged by different kinds of pathogens trying to invade and colonize their tissue. During the course of evolution both hosts and pathogens have evolved highly specialized and sophisticated mechanisms for an efficient defense and invasion, respectively, which is described in the so-called zigzag model (Fig. 1.10A; Zvereva *et al*., 2012). Starting with the release of pathogen/microorganism-associated molecular patterns (PAMPs/MAMPs), such as flagellin or chitin, or damage-associated molecular patterns (DAMPs) derived from the host, such as cellulose monomers, the plant senses these molecules when they bind to PM-associated receptor-like kinases (RLKs) that intracellularly activate signal transduction cascades evoking defense responses against the invading pathogen (Fig. 1.10B; Wirthmueller *et al*., 2013). Several bacteria, fungi and oomycetes have evolved specific effector molecules that are able to suppress this so-called pattern recognition receptor (PRR)-mediated immune response, a mechanism that is called effector-triggered susceptibility (ETS). However, the host plant contains specific intracellular receptors for the recognition of effector proteins. These receptors consist of a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain and undergo conformational changes upon effector-binding, which finally leads to NB-LRR-mediated immunity of the host plant. Many pathogens have developed novel effector molecules for the suppression of this immunity response so that the plant has to find new ways for the defense against fungal or bacterial invaders. These continuous adaptations and modifications of effector and receptor proteins are thought to be the driving forces during evolution for the development of successful invasion strategies on the pathogen side and efficient defense mechanisms on the host side.

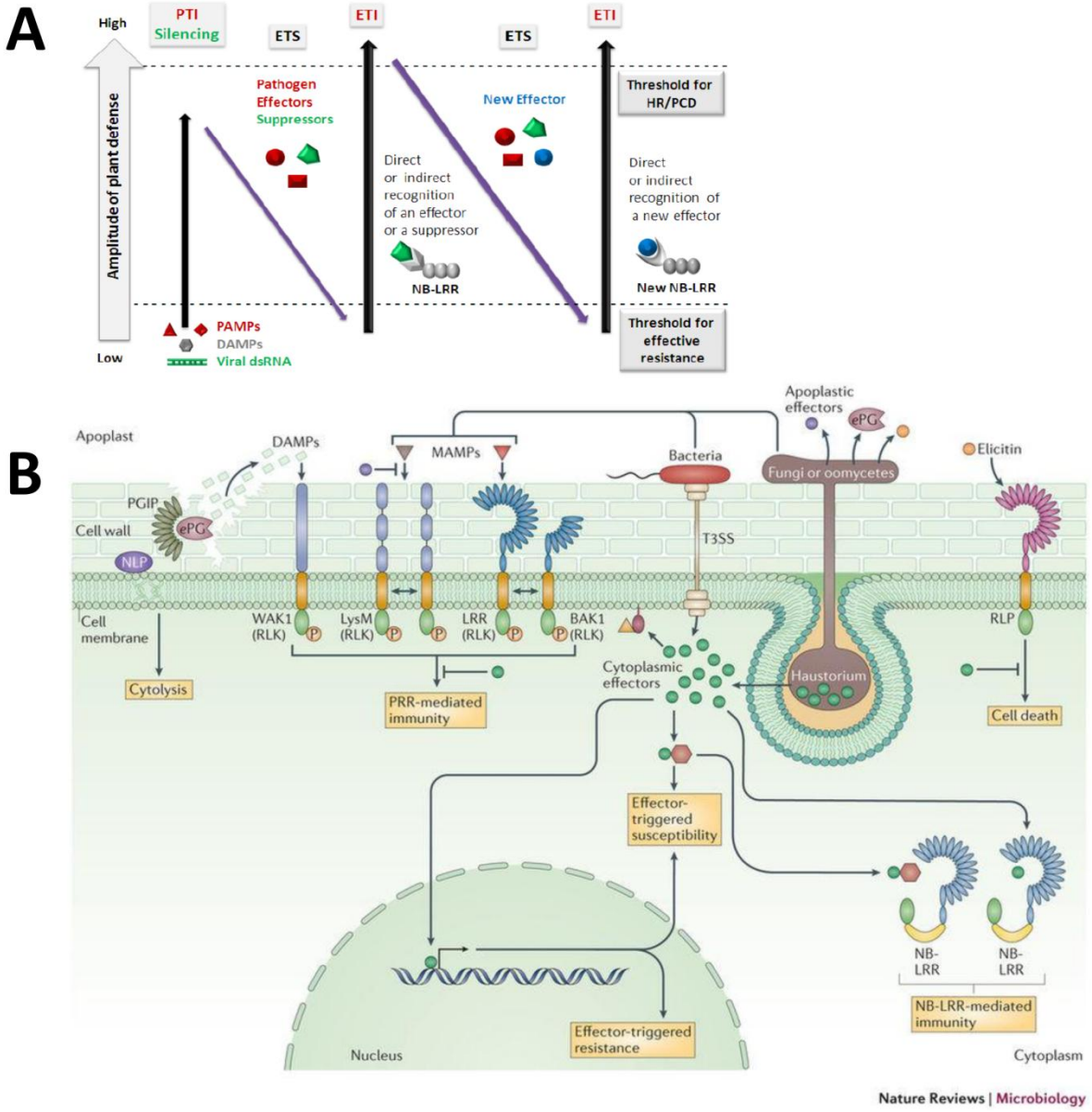

Figure 1.10 Interactions of pathogens with their host plant | (a) Zigzag model describing the evolution of plant-pathogen interactions. PAMPs or DAMPs are released at the interaction surface and evoke immune responses in the host plant, which can be suppressed by secreted pathogen-derived effector molecules. In turn the plant can perceive these effectors with specialized receptors for the reactivation of immune responses. Many pathogens have developed further effector proteins for the inhibition of this second plant response and in the host plant novel receptors evolved as defense mechanism and so on and so forth. Abbreviations: PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; PTI, pathogen triggered immunity; ETS, effector triggered susceptibility; ETI, effector-triggered immunity; NB-LRR, nucleotide-binding leucine rich repeat domain receptor; HR, hypersensitive response; PCD, programmed cell death (source: Zvereva, A. S., & Pooggin, M. M. (2012). Silencing and innate immunity in plant defense against viral and non-viral pathogens. Viruses, 4(11), 2578-2597). (b) Scheme of cellular components involved in host-pathogen interactions. Some pathogens disturb the structure of the host's membrane in order to promote cytolysis for an efficient colonization of the plant tissue, while biotrophic pathogens form haustoria for an interaction with the living plant tissue. The plant's defense starts with the perception of released DAMPs or MAMPs by specific PM-associated RLKs leading to the intracellular activation of immune responses. Many pathogens further secrete effector proteins either via type III secretory systems

(bacteria) or via the haustorial interface (oomycetes, fungi) into the host cell for the suppression of immune responses or elicitin-induced cell death. These effectors molecules can in turn be recognized by intracellular NB-LRR receptors of the plant to prevent susceptibility. Abbreviations: ePG, endo-polygalacturonases; PGIP, polygalacturonase inhibiting protein; MAMPs, microorganism-associated patterns; WAK1, wall-associated receptor kinase 1; PRR, pattern recognition receptor; RLK, receptor-like kinase; RLP, receptor-like protein; LRR, leucine-rich repeat domain; BAK1, BRI1-associated receptor kinase 1; NB, nucleotide-binding; T3SS, type III secretion system (source: Wirthmueller, L., Maqbool, A., & Banfield, M. J. (2013). On the front line: structural insights into plant-pathogen interactions. Nature Reviews Microbiology, 11(11), 761-776.)

While necrotrophic fungi, such as *Botrytis cinerea* or *Fusarium oxysporum*, promote cell death in order to use the nutrients of the dead plant tissue for their growth, biotrophic pathogens, such as *Blumeria graminis* or *Puccinia graminis*, rely on living plant tissue for an efficient colonization of the host (Govrin & Levin, 2000; Nowara *et al.*, 2010). The latter establish feeding structures called haustoria for the uptake of nutrients from the plant as well as the delivery of effector molecules into the plant cell (Whisson *et al*., 2007). The formation of these feeding structures is a very complex process and requires the penetration of the plant's cell wall. This is often achieved either by enzymatic degradation of the structural cellulose/hemicelluloses polymers and/or by the mechanical force of a penetration peg derived from fungal appressoria (Tucker *et al*., 2001). Afterwards, the fungal hyphae are able to invaginate the PM of the host plant in order to create a large haustorial interface required for an efficient supply of the pathogen with nutrients and for the delivery of effector molecules into the host cytoplasm.

1.8 Lipid signaling and cellular stress responses

Stimulation by various kinds of external biotic factors, for example derived from invading fungal pathogens, or abiotic factors, such as mechanical forces, certain chemicals or temperature shifts, leads to the activation of cellular response mechanisms in every organism. The corresponding stimuli are perceived by channels or specialized receptors at the plasma membrane (PM), transmitting the signal to intracellular components to initiate signal transduction cascades, which in the end elicit cellular responses for the adaptation to the changing environment (Bradshaw & Dennis, 2009). Typically these signal transduction cascades involve the generation of second messenger molecules, which are often derived from membrane lipids, *e.g.* diacylglycerol (DAG), phosphatidic acid (PA) or inositol trisphosphate (IP_3) (Spiegel *et al*., 1996). The latter gets released from the PM after cleavage of the structural lipid phosphatidyl inositol-(4,5)-bisphosphate (PI(4,5)P2) by phospholipase C (PLC),

while DAG remains in the membrane. Upon binding to its receptors IP_3 can stimulate the release of Ca^{2+} ions from internal stores like the endoplasmic reticulum (ER) or vacuoles and thereby activate diverse Ca^{2+}-dependent responses (Hanson *et al.*, 2004). In addition, DAG attracts protein kinase C (PKC), a central regulator of cell proliferation and signal transduction, to the membrane and consequently enhances its activity (Newton, 1995). Interestingly, PKC does not exist in plants and it is thought that related plant-specific protein kinases, which require PA instead of DAG, can functionally replace PKC by activating the corresponding signaling pathways (Subramaniam *et al*., 1997).

Not only PLC but also PLD and PLA are important mediators in the generation of cellular responses to external stimuli. Especially in plants as sessile organisms lipid modifying enzymes are crucial for rapid adaptations to changing environmental conditions and for bringing about hormone responses, which is reflected in the huge number of their isoforms in plants (Wang *et al*., 2002). PLD cleaves off the polar head groups of several PLs with preference for phosphatidylcholine (PC) and thereby produces PA, the most prominent lipid signaling molecule in plants. Several reports have proven the activation of diverse PLD isoforms during abiotic and biotic stress. For example, in rice a knockdown of PLDβ1 induces the overproduction of reactive oxygen species (ROS) and the expression of pathogenicity-related (PR) genes in the absence of pathogen infection leading to enhanced disease resistance (Yamaguchi *et al*., 2009). The corresponding PLD isoform in *A. thaliana* seems to have overlapping functions as a deficiency of its activity leads to the same physiological effects (Zhao *et al*., 2013). It is further suggested that PLDα and PLDδ may regulate plant defense by a modification of microtubule organization with severe impact on the routing of secretory proteins to the site of fungal penetration (Zhao, 2015). The complex plant-pathogen interaction network is additionally regulated by the hormone system. During the last years it has become clear that not only the classical defense hormones salicylic acid (SA) and jasmonic acid (JA) are involved in this network but also all other phytohormones, such as gibberellins, auxins, abscisic acid (ABA) or ethylene (Pieterse *et al*., 2009). The interplay of all these hormones is very often regulated by PLD-PA mediator complexes that evoke the corresponding cellular responses (Zhao, 2015). Not only PLD but also PLA fulfills important functions during the interaction with pathogens. PLA removes fatty acid moieties either at the sn1 or sn2 position of PLs, which is for example in animals required for the biosynthesis of prostaglandins from arachidonic acid (Irvine, 1982). In plants, PLA can liberate polyunsaturated FAs from galactolipids in plastids required for JA formation during the plant's defense response (Dave & Graham, 2012).

The hypothetical model in Fig. 1.11 provides an overview on PL signaling in plants (Munnik *et al*., 1998). Even if this report was published 20 years ago, the model is still valid and summarizes clearly the most important factors that are involved in PL signaling including the physiological processes

regulated by lipid intermediates. Starting with the receptor-mediated perception of external signals, lipid-modifying enzymes are activated and recruited to the PM for the conversion of structural membrane lipids into second messenger molecules. Not only the before mentioned PLases but also phosphatidic acid kinase (PAK) and phosphatidyl inositol-3-phosphate kinase (PI3K) play essential roles in the formation of second messengers, which are in this case diacylglycerol pyrophosphate (DGPP) and phosphatidyl inositol-3,4-bisphosphate (PI(3,4)P2), respectively. Although their precise cellular targets are often unknown, the evidence demonstrating their importance as signaling molecules in diverse cellular processes, *e.g.* lipid recycling, vesicular transport or endocytosis, is steadily increasing (Munni *et al.*, 1998; Balboa *et al.*, 1999; Posor *et al.*, 2013).

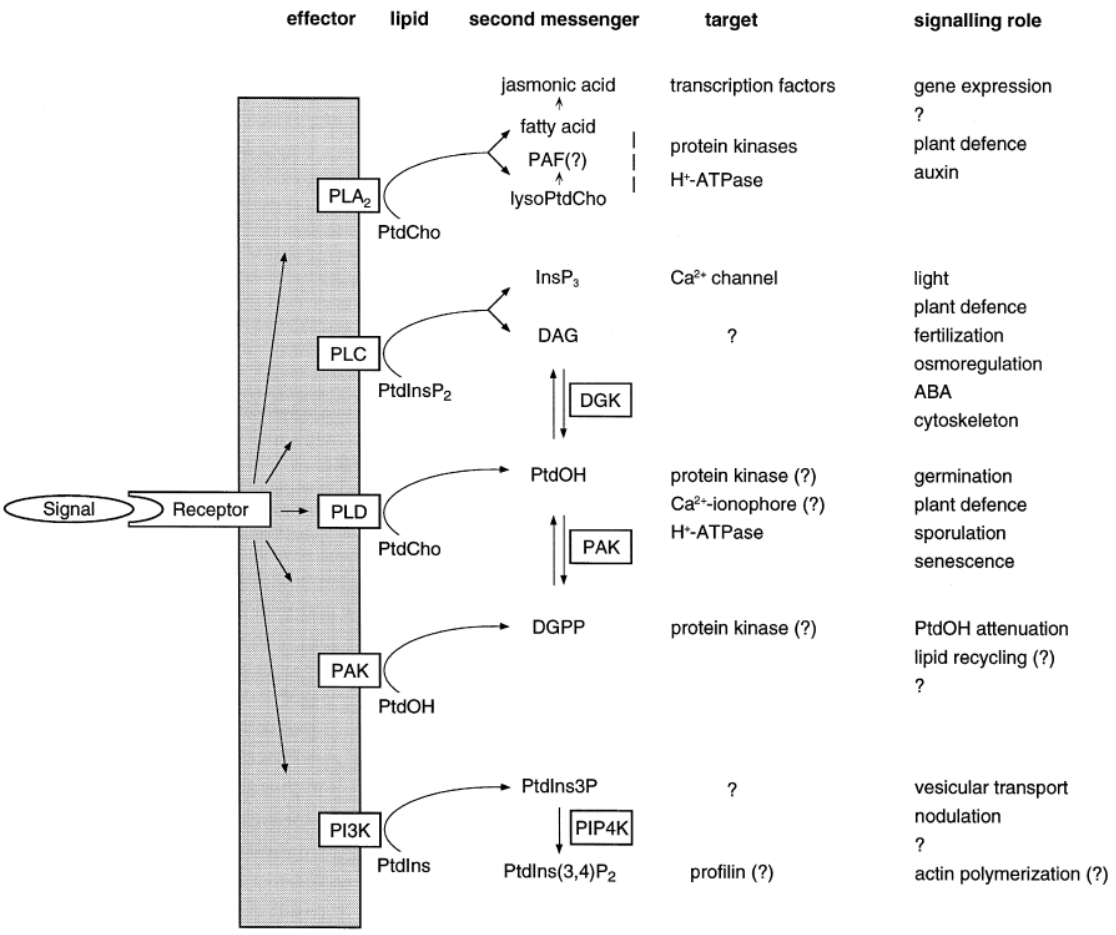

Figure 1.11 Hypothetical model of PL signaling in plants according to Munnik *et al.*, 1998 | External signals, *e.g.* hormones or pathogen derived PAMPs are perceived by PM-coupled receptors, which activates PL-modifying enzymes (PLA2, PLC, PLD, PAK, PI3K) that convert structural lipids into second messengers. These molecules then activate their cellular targets, such as protein kinases or Ca^{2+} channels, which finally results in diverse physiological responses. Abbreviations: PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PAK, phosphatidic acid kinase; PI3K, phosphatidylinositol-3-phosphate kinase; PAF, platelet-activating factor; lysoPtdCho, lyso-phosphatidylcholine; $InsP_3$, inositol-trisphosphate; DAG,

diacylglycerol; PtdOH, phosphatidic acid; DGPP, diacylglycerolpyrophosphate; PtdIns3P, phosphatidylinositol-3-phosphate. (source: Munnik, T., Irvine, R. F., & Musgrave, A. (1998). Phospholipid signalling in plants. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1389(3), 222-272.)

Cellular responses upon stimulation by external factors not only involve PLs as signaling molecules but also sphingolipids. This lipid class is characterized by a vast spectrum of potential chemical modifications including glycosylations, hydroxylations and phosphorylations and both the long-chain base (LCB) and the very long-chain fatty acid (VLCFA) moieties can vary in chain length and degree of desaturation (Fig. 1.12; Michaelson *et al*., 2016). This structural variability is thought to be the reason for their distinct biological activities and explains at the same time the difficulty of their analysis. Most reports focus on the role of specific sphingolipids involved in cold acclimation, salt tolerance, disease resistance or adaptations to hypoxic conditions (Chen *et al*., 2012; Wu *et al*., 2015; Xie *et al*., 2015). On the one hand, desaturation of the LCB or the VLCFA moiety significantly affects the membrane fluidity, which is a crucial factor for tolerating these adverse conditions. On the other hand, phosphorylated sphingolipids, especially (phyto)sphingosine-1P and ceramide 1-phosphate, act as signaling molecules that for example promote stomatal closure via G protein-coupled receptor pathways or activate cold stress responses (Ng *et al*., 2001; Coursol *et al*., 2003; Chen *et al*., 2012). Moreover, a shift of the ceramide/ceramide 1-phosphate ratio towards the unphosphorylated form promotes ROS accumulation and programmed cell death (PCD) in plants with further consequences for the interaction with biotrophic and necrotrophic pathogens (Brodersen *et al*., 2002; Liang *et al*., 2003).

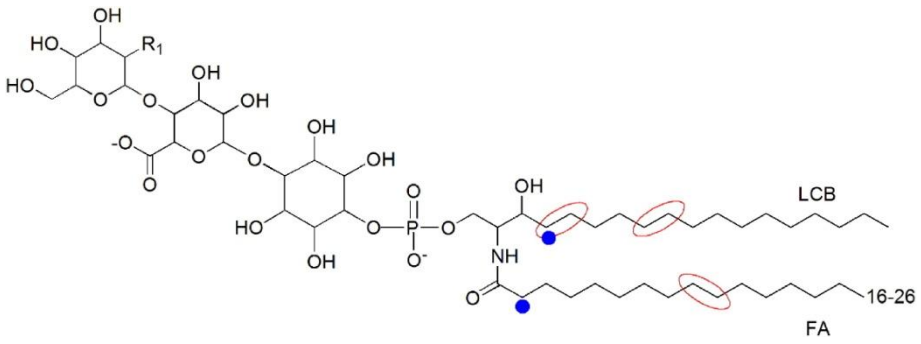

Figure 1.12 Basic structure of sphingolipids | Sphingolipids consist of a sphingoid long-chain base (LCB, 16-20 C-atoms) that is amide-linked to a long-chain or very long-chain fatty acid (VLCFA, 16-30 C-atoms) moiety. Blue dots indicate potential hydroxylation sites and red lines potential desaturations. (source: Michaelson, L. V., Napier, J. A., Molino, D., & Faure, J. D. (2016). Plant sphingolipids: Their importance in cellular organization and adaption. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1861(9), 1329-1335.)

1.9 Aim of the thesis

The ABC transporter Lr34res is known to confer partial, durable and broad-spectrum resistance against several biotrophic pathogens in wheat and in related grass species expressing the gene. Moreover, side-effects such as premature senescence and the promotion of stress-responsive gene expression are observed in plants with a high *Lr34res* expression level. The aim of the thesis was to elucidate the molecular mechanisms behind the Lr34res-mediated pathogen resistance in an attempt to link them to the observed side-effects.

This work demonstrates that the ABC transporter is associated with the plasma membrane where it modifies the distribution of several phospholipids. Follow-up experiments deal with the consequences of Lr34-mediated plasma membrane remodeling for cellular lipid metabolism that is closely connected to abiotic and biotic stress responses. Another aspect of the thesis is the establishment of suitable expression systems for a reconstitution of the ABC transporter in liposomes required for a detailed analysis of its activity and substrate affinity.

2 The wheat ABC transporter Lr34 modifies the lipid environment at the plasma membrane

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Running title: ABC transporters and phospholipids

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2.1 Abstract

Phospholipids (PLs) are emerging as important factors that initiate signal transduction cascades at the plasma membrane. Their distribution within biological membranes is tightly regulated *e.g.* by ATP-binding cassette transporters, which preferably translocate PLs from the cytoplasmic to the exoplasmic membrane leaflet and are therefore called PL-floppases. Here we demonstrate that a plant ABC transporter, Lr34 from wheat (*Triticum aestivum*), is involved in plasma membrane remodeling characterized by an intracellular accumulation of phosphatidic acid and enhanced outward translocation of phosphatidylserine. In addition, the content of phosphatidylinositol 4,5-bisphosphate in the cytoplasmic leaflet of the plasma membrane was reduced in the presence of the ABC transporter. When heterologously expressed in *Saccharomyces cerevisiae*, Lr34 promoted oil body formation in a mutant defective in PL transfer in the secretory pathway. Our results suggest that PL redistribution by Lr34 potentially affects the membrane-bound proteome and contributes to the previously reported stimuli-independent activation of biotic and abiotic stress responses and neutral lipid accumulation in transgenic *Lr34*-expressing barley plants.

2.2 Introduction

ATP-binding cassette (ABC) transporters constitute a large and diverse family of membrane proteins that use the energy provided by ATP hydrolysis to pump various compounds against steep concentration gradients. They occur as full-size or half-size transporters and form an active unit consisting of two transmembrane and two nucleotide binding domains. The ubiquitous presence of ABC transporters in almost all organisms highlights their importance for cellular metabolism. Typically, they play a major role in extruding a multitude of cytotoxic compounds like heavy metal ions or xenobiotics (1-2); however, their true physiological function goes far beyond as deduced from the fact that malfunction of certain ABC transporters can evoke drastic physiological effects. For instance, the human Tangier disease, characterized by the absence of high-density lipoprotein, can be traced back to a defect in *Hs*ABCA1, a member of the human A subfamily of ABC transporters. In addition, mutations in *Hs*ABCA4 result in delayed dark adaptation in the retina by a reduced all-trans-retinaldehyde clearance, a characteristic feature of Stargardt disease (2). The strong association with lipid metabolism suggests that certain lipids might directly serve as transport substrates. Indeed, several reports have demonstrated a role of numerous ABC transporters in the rearrangement of certain lipids within the membrane bilayer. Depending on the direction of movement the translocator is either called flippase (from the exoplasmic to the cytoplasmic leaflet) or floppase (from the cytoplasmic to the exoplasmic leaflet). A prominent example is the pleiotropic drug resistance type transporter PDR5 from *Saccharomyces cerevisiae,* which was characterized as a phosphatidylethanolamine (PE) floppase besides its function in extruding a diverse set of cytotoxic compounds (3). In addition, the human pathogen *Leishmania* contains a half size ABCG transporter, which is responsible for the exposure of phosphatidylserine (PS) on its plasma membrane (PM) (4). The same effect has been observed in human gastric carcinoma cells, where the overexpression of the breast cancer resistance protein BCRP (ABCG2) leads to increased PS exposure (5). Moreover, the full size ABCG transporters CDR1-3p from the pathogenic fungus *Candida albicans* were characterized as phospholipid (PL) translocators with varying transport direction and broad substrate specificity (6).

The wheat ABC transporter Lr34, a member of the G subfamily, is known to confer partial, durable and broad-spectrum resistance against several biotrophic fungi such as powdery mildew, leaf rust or stem rust (7). In the wheat gene pool, the *Lr34* gene is present in two different alleles, called *Lr34sus* and *Lr34res,* which are characterized by their susceptible and resistant phenotype, respectively, to biotrophic fungal pathogens in the corresponding wheat varieties. These two alleles differ at two positions within the coding region for the first transmembrane domain leading to a phenylalanine deletion and a conversion of tyrosine to histidine in Lr34res. It is still unclear whether the phenotypic

differences are caused by varying protein levels or distinct biochemical functions of the two transporter versions. Recent reports have demonstrated that *Lr34res* can be functionally transferred to related crop species like barley, rice or maize making them partially resistant against their specific pathogens (8-10). However, strong expression levels of the transgene correlate with the development of early leaf tip necrosis as an indicator of senescence (8) and strong triacylglycerol (TAG) accumulation in transgenic barley plants (11). Moreover, transcriptomic data of *Lr34res*-expressing barley have revealed the upregulation of numerous stress- and pathogenicity-related (PR) genes in the absence of fungal infection (12, 13). In order to explain all these observations it is essential to understand how the ABC transporter functions at the molecular level.

The fact that several stress-responsive genes are upregulated in *Lr34res*-expressing plants in the absence of pathogens (12-13) suggests that the activity of the transporter mimics stress exposure likely by modulating cellular metabolism. A common feature of hormone and pathogen defense signaling pathways is the activation of certain phospholipases (PLases) upon the perception of external signals (14). These enzymes convert membrane lipids into signaling compounds like phosphatidic acid (PA), diacylglycerol (DAG) or inositoltriphosphate (IP_3), which affects downstream PL-interacting factors in their subcellular localization and thus their activity. A prominent example in plants is the protein phosphatase ABI1 from *Arabidopsis thaliana*, which binds to PA in the PM upon phospholipase D (PLD) activation and thereby loses its suppressor function in the ABA signaling pathway (15). Other reports showed that PLDβ1 has a major role in modulating defense responses against bacterial and fungal pathogens through the generation of PA (16). The aim of this work was to address whether the ABC transporter Lr34 affects membrane properties that might lead to the activation of PL-mediated stress signaling in the absence of external factors. We demonstrate that Lr34 functions as a translocator for PA and PS in transgenic barley and changes the distribution of PS and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) in the PM of tobacco BY2 cells. When heterologously expressed in *S. cerevisiae* the ABC transporter enhanced PS and reduced PE exposure at the PM. Finally, the presence of Lr34 promoted the accumulation of neutral lipids in oil bodies in a *S. cerevisiae* mutant strain defective in PL transfer. Together, these data suggest that Lr34 plays a pivotal role in redistributing certain membrane PLs with impact on overall cellular lipid metabolism.

2.3 Results

2.3.1 Lr34res is present in the PM fraction when heterologously expressed in tobacco BY2 cells

Pleiotropic drug resistance (PDR)-type transporters are usually found in the plasma membrane (17-19). As a prerequisite for subsequent functional studies it was necessary to confirm the presence of Lr34res in the plant PM. Therefore, the total membrane fraction from tobacco BY2 cells expressing HA-tagged Lr34res was isolated and fractionated on a linear density gradient. Immunolabeling of the fractions with antibodies directed to specific proteins residing at different intracellular membrane compartments demonstrated that the distribution of HA-Lr34res correlated with the PM H^+-ATPase marker (Fig. 1A and B) indicating that Lr34res is indeed associated with the plant PM.

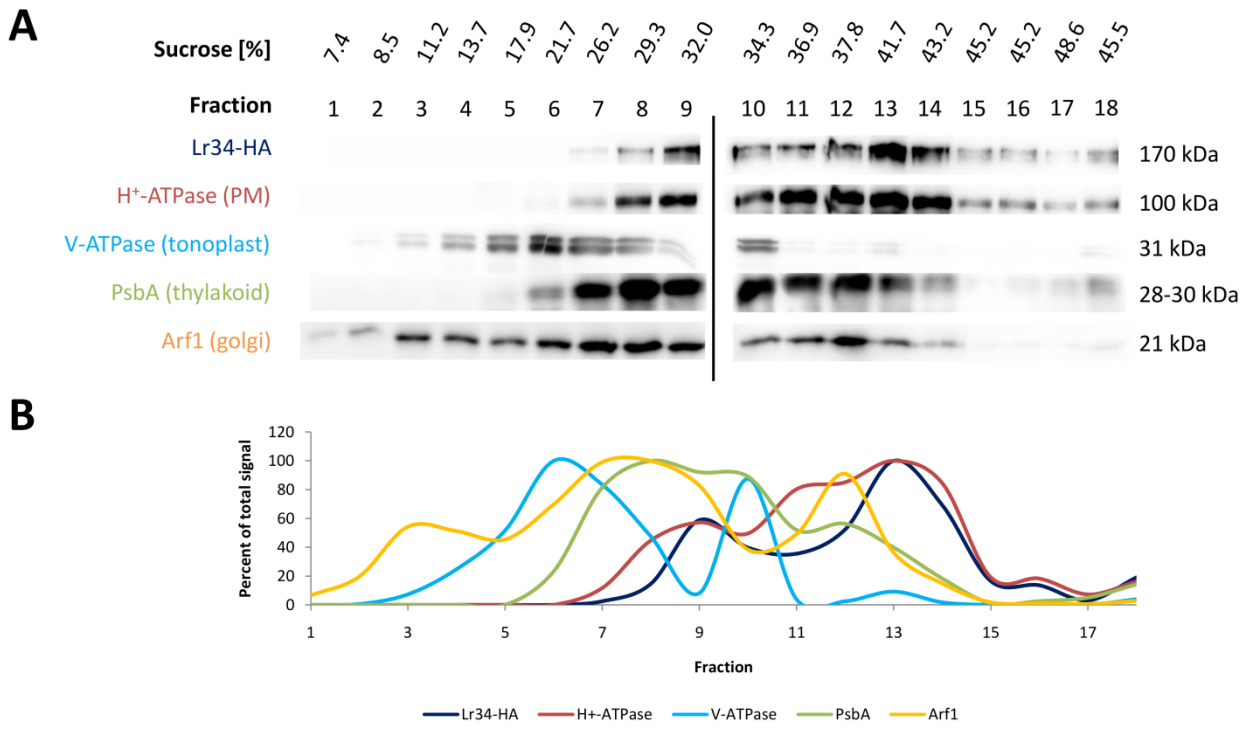

Figure 1 Total membrane fractionation of BY2 cells expressing HA-tagged Lr34res protein | (a) Fractions ranging from 7.4-48.6 % (w/v) sucrose were harvested after ultracentrifugation and tested for the presence of HA-Lr34res and marker proteins of the PM (H^+-ATPase), the tonoplast (V-ATPase), thylakoid membranes (PsbA) and Golgi membranes (Arf1) by Western blotting with specific antibodies. (b) Quantification of signal intensities after blot development. For each protein values are relative to the fraction with the strongest signal, which was set to 100%.

2.3.2 Lr34res promotes PA inward and PS outward translocation in barley protoplasts

In the next step the activity of the ABC transporter was investigated that might be responsible for the Lr34res-mediated resistance against various biotrophic pathogens. Based on numerous reports demonstrating a role of ABC transporters in PL metabolism (3-6), as well as the observed phenotypes in plants expressing the resistance gene (8, 9), we hypothesized that Lr34res might have a PL flippase

or floppase activity with impact on the distribution of certain PLs within the membrane. To study this putative function *Lr34res*-expressing (BG9) and the corresponding non-transgenic (sib) barley plants (see ref. 8) were used to isolate protoplasts. These were then incubated with fluorescence-labelled (NBD) PLs to investigate their incorporation in a time-dependent manner. The uptake rate of NBD-PC, NBD-PE and NBD-PG was similar between sib and BG9 protoplasts at all tested time points (Fig. 2). By contrast, NBD-PA strongly accumulated in transgenic BG9 protoplasts after 10 minutes, whereas NBD-PS-uptake was significantly reduced.

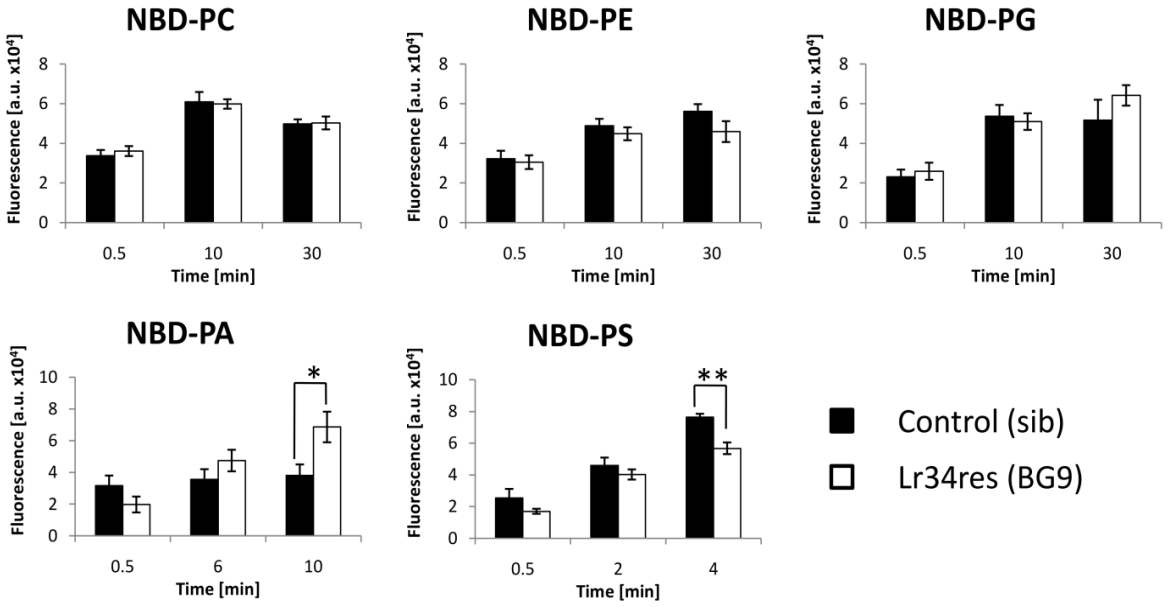

Figure 2 Time dependent incorporation of different NBD-labelled PLs in barley protoplasts expressing *Lr34res* | Leaf mesophyll protoplasts were isolated from *Lr34res*-expressing (BG9) and the corresponding non-transgenic (sib) barley plants and incubated with NBD-phosphatidic acid (NBD-PA), NBD-phosphatidylethanolamine (NBD-PE), NBD-phosphatidylglycerol (NBD-PG), NBD-phosphatidylcholin (NBD-PC) or NBD-phosphatidylserine (NBD-PS). At the indicated time points samples were taken and the incorporated fluorescence quantified. Values are means of arbitrary units (a.u.) ± s.e.m (n=4-6), *P≤0.05, **P≤0.01, according to Student's t-test.

2.3.3 Lr34res reduces PS and PI(4,5)P2 levels in the cytoplasmic PM leaflet of tobacco BY2 protoplasts

The advantage of using NBD-labelled PLs is the easy detection and quantification of the emitted fluorescence. However, their physical properties slightly differ from natural lipids, which potentially affects their behavior in the hydrophobic membrane environment. To avoid this possible problem, protoplasts from control and transgenic *Lr34res*-expressing tobacco BY2 cells were transformed with PL-specific biosensors to visualize the intracellular distribution of selected native membrane lipids.

In agreement with previous reports (20) the phosphatidylinositol 3-phosphate (PI(3)P)-binding, red fluorescent protein (RFP)-tagged biosensor P18R was enriched in intracellular membranes both in the presence and absence of Lr34res (Fig. 3A). The phosphatidylinositol 4-phosphate (PI(4)P)-binding biosensor P21R and the corresponding citrine-tagged version (P21Y) showed the strongest signal at a membrane that could be stained with FM4-64 at the periphery of the protoplast (Fig. 3A and B). This is consistent with the normal localization of PI(4)P at the PM (20). While the ABC transporter did not affect the subcellular destination of the two monophosphorylated inositides, the phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-binding biosensor P24R did not label the PM anymore under Lr34res expression but was instead restricted to intracellular, vacuole-like structures. A PS biosensor (Lact-C2) was present at the PM and endomembranes independently of Lr34res expression. However, an accumulation of this biosensor in dot-like structures was observed in Lr34res-expressing protoplasts in contrast to the control.

For a verification of these observations, the ratio of the PM/total signal was calculated for the PI(4,5)P2 and PS biosensors. This indeed confirmed a significantly lower fluorescence signal at the PM for the PI(4,5)P2 and PS biosensors in the presence of Lr34res (Fig. 3C) suggesting that these lipids accumulate less at the cytoplasmic PM leaflet.

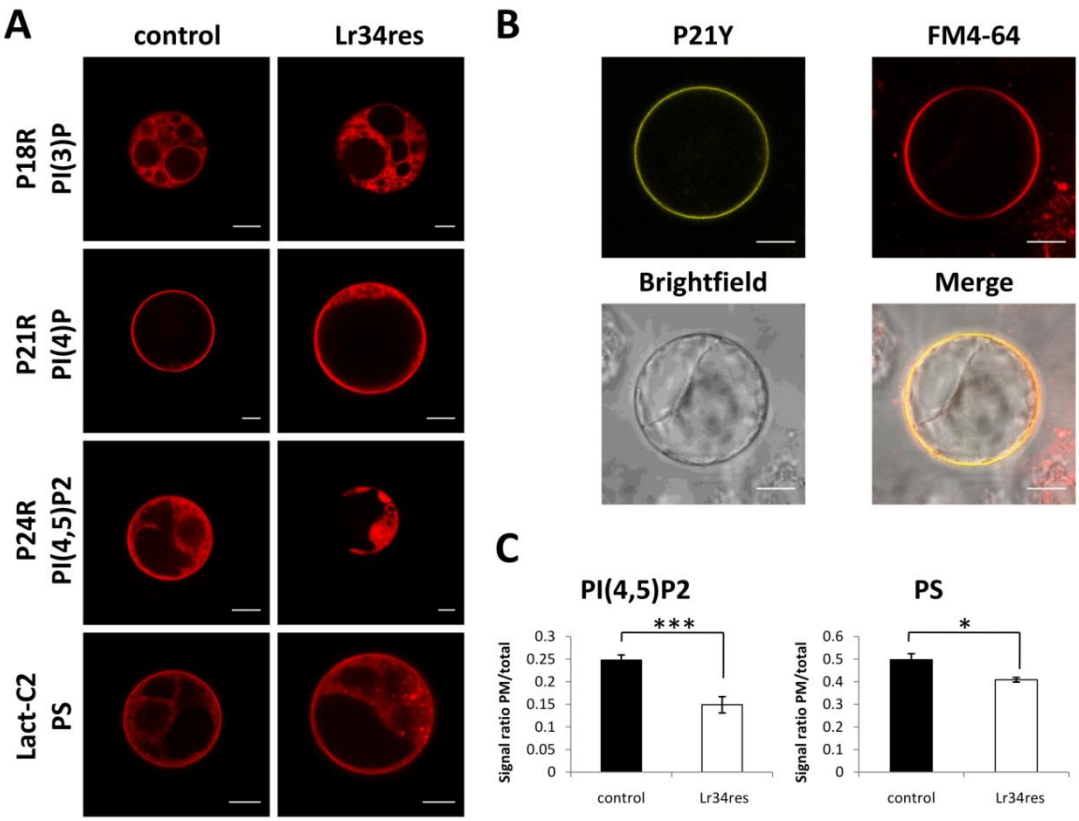

Figure 3 Subcellular localization of fluorescent PL biosensors in BY2 protoplasts | (a) Red fluorescent protein **(**RFP)-tagged biosensors for PS (derived from the C2 domain of lactadherin), PI(4)P (P21R, ref. 20), PI(4,5)P2 (P24R, ref. 20) and PI(3)P (P18R, ref. 20) were transiently expressed in transgenic control and *Lr34res* transformed BY2 protoplasts. Pictures were taken 16-18 h after transfection. (b) Colocalization of the citrine-tagged version of the PI(4)P biosensor (P21Y) with FM4-64 in BY2 control protoplasts. In this case, P21Y was selected instead of P21R to avoid the overlap of RFP and FM4-64 fluorescence emission spectra. (c) Calculation of the ratio of PM-derived to total signal for PI(4,5)P2 and PS biosensors in control and Lr34res BY2 protoplasts. Values are means ± s.e.m (n≥8), *P≤0.05, ***P≤0.001, according to Student's t-test. Scale bars, 10 µm**.**

2.3.4 Lr34res and Lr34sus enhance PS and reduce PE exposure at the PM in *Saccharomyces cerevisiae*

To confirm the role of Lr34 as a translocator for certain PLs and to investigate potential differences in activity between the two transporter versions, Lr34sus and Lr34res, the protein was heterologously expressed in a wild type *S. cerevisiae* strain in order to perform growth assays that use toxins to probe for the translocation of specific endogenous PLs at the PM. In addition to the two native Lr34 proteins, Lr34sus and Lr34res, inactive versions bearing mutations of the critical amino acids in all four Walker domains (Lr34sus/resW, table S1) were used as controls besides the empty vector because these domains are essential for the hydrolysis of ATP in all ABC transporters.

Under standard conditions the expression of an active Lr34 transporter impaired growth in yeast cells, whereas the inactive Lr34 versions did not cause any effect even when slightly higher protein expression levels were achieved (Fig. 4A and B).

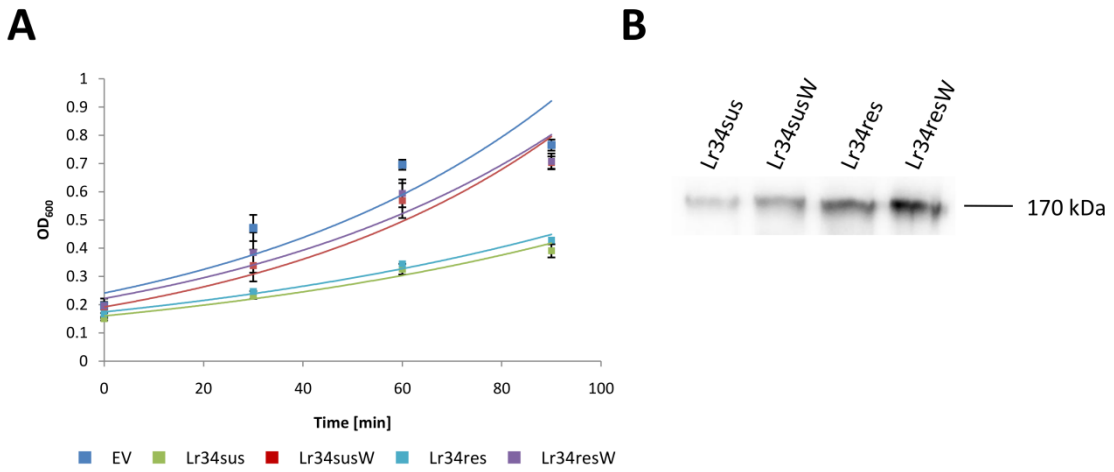

Figure 4 Growth behavior of *S. cerevisiae* wild type strain BY4741 expressing different Lr34 versions | (a) Cells were either transformed with an empty vector (EV), or plasmids for the expression of Lr34sus, inactive Lr34sus (Lr34susW), Lr34res or inactive Lr34res (Lr34resW) and grown in selective SD media under standard growth conditions. OD_{600} was measured at the

indicated time points during the exponential growth phase. Values are means ± s.e.m (n≥5). (b) Immunoblot analysis of microsomal yeast fractions expressing different HA-tagged versions of Lr34.

Next, the growth behavior of *Lr34*-expressing yeast cells was investigated in the presence of specific toxins. The cyclic depsipeptide duramycin specifically binds PE upon exposure to the outer leaflet of the PM and forms pores in the membrane that induce cell death (21). Therefore, any floppase activity resulting in increased PE exposure should enhance the drug sensitivity of the cells, whereas a PE flippase would evoke the opposite effect. Interestingly, duramycin application altered the general growth behavior of yeast cells, indicated by a constant linear growth and loss of clearly distinguishable growth phases (Fig. S1). Moreover, when growth inhibition was calculated, cells expressing Lr34sus and especially Lr34res were more resistant to the drug and consequently grew significantly better than the corresponding controls at 5 and 10 µM duramycin (Fig. 5), pointing to a PE flippase activity of both Lr34 versions.

To get further insights on the PL specificity of the ABC transporter, the growth of cells expressing the different Lr34 versions was also tested in media containing miltefosine. This compound is a structural analogue of lyso-PC and develops its toxic effect upon transport into the cell (22). If Lr34 were able to extrude miltefosine, yeast cells expressing the ABC transporter should be less sensitive to this drug. Apparently, even small toxin concentrations altered the growth behavior in a similar way as duramycin. However, when comparing the growth inhibition rates it was asserted that in this case neither *Lr34sus* nor *Lr34res* expression significantly altered the drug sensitivity of the yeast cells, suggesting that no import or export of miltefosine occurred through the transporters.

Similarly, cells were tested for growth in the presence of the PS-binding cyclic depsipeptide papuamide A, which develops its toxic effect by the same mechanism as duramycin (23, 24). The growth of *Lr34sus*- and *Lr34res*-expressing cells was significantly impaired compared to cells expressing the corresponding inactive transporter versions or containing the empty vector already at 0.6 µg/ml papuamide A. This effect persisted when increasing the drug concentration to 1 µg/ml.

In summary, Lr34sus and Lr34res enhanced the tolerance to duramycin, suggesting that PE is translocated to the inner side of the yeast PM. The opposite effect was observed for papuamide A, indicating a role of Lr34sus and Lr34res in PS exposure. In accordance with the protein expression levels (Fig. 4B) these effects were slightly more pronounced for Lr34res than for Lr34sus. Finally, unaltered miltefosine sensitivity suggests that lyso-PC does not serve as substrate for Lr34.

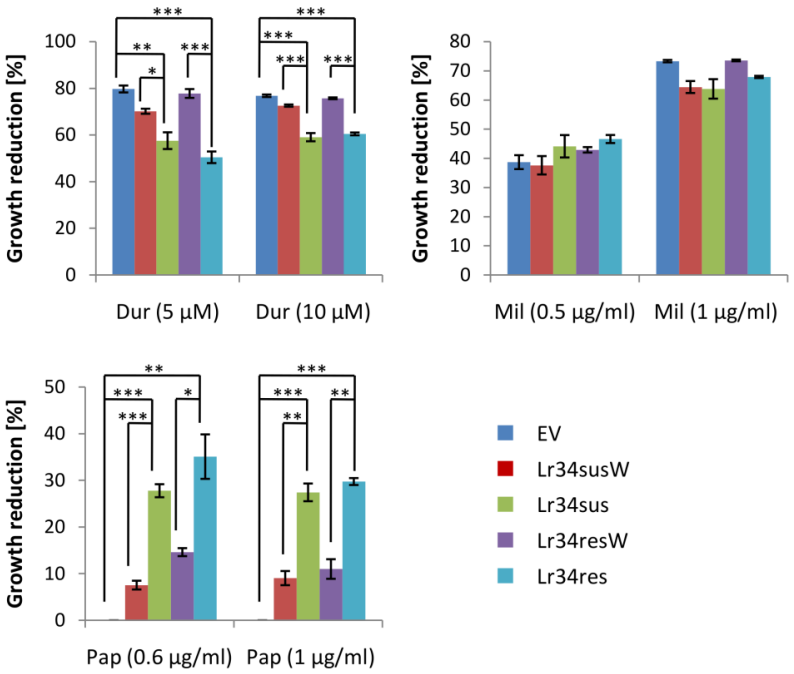

Figure 5 Drug sensitivity test of yeast BY4741 cells expressing different Lr34 versions | Growth inhibitory effects of the cytotoxic drugs duramycin (Dur), miltefosine (Mil) and papuamide A (Pap) on yeast cells containing an empty vector (EV) or expressing inactive Lr34sus (Lr34susW) or Lr34res (Lr34resW) or the active protein versions Lr34sus or Lr34res were determined at two different drug concentrations. Values are means ± s.e.m. (n=4-6), *P≤0.0167, **P≤0.0033, ***P≤0.00033, according to one way ANOVA and post-hoc t-tests with Bonferroni corrections.

2.3.5 Expression of Lr34res promotes oil body formation in a PL transfer-deficient *Saccharomyces cerevisiae* mutant strain

The previously reported triacylglycerol accumulation in leaves of *Lr34res*-expressing barley plants (11) suggests a potential link between the PL flippase or floppase activity of Lr34res and overall lipid metabolism. As Lr34res apparently not only translocates PLs in the PM of plants but also in the PM of yeast, it could be assumed that enhanced neutral lipid storage might also be induced by the ABC transporter *in fungo*. Instead of using wild type *S. cerevisiae* strains that hardly accumulate any lipids under standard growth conditions, the *Sec14*^{ts} PL transfer protein mutant CTY1-1A showing constitutively enhanced oil body formation for the storage of accumulating neutral lipids was selected for the following studies. This mutant is characterized by a defect in the PL transfer protein Sec14, which is necessary for efficient vesicle budding and fusion events between the trans-Golgi network and the PM. Because a deletion of the *Sec14* gene is lethal, a mutation was introduced in the coding sequence that renders the yeast cells temperature sensitive due to impaired PL transfer activity by the mutated Sec14 protein (25). In order to visualize oil bodies and quantify the

intracellular oil content, the lipophilic dye Nile red was applied that is only fluorescent when embedded in a hydrophobic environment typically found in oil bodies (26). Expression of Lr34res but not Lr34sus significantly increased the number and the size of these lipid reservoirs (Fig. 6A and B). Since expression levels of both Lr34 versions were similar in this mutant (Fig. 6C), a different activity of the two transporters can be assumed to be the cause for the accumulation of neutral lipids.

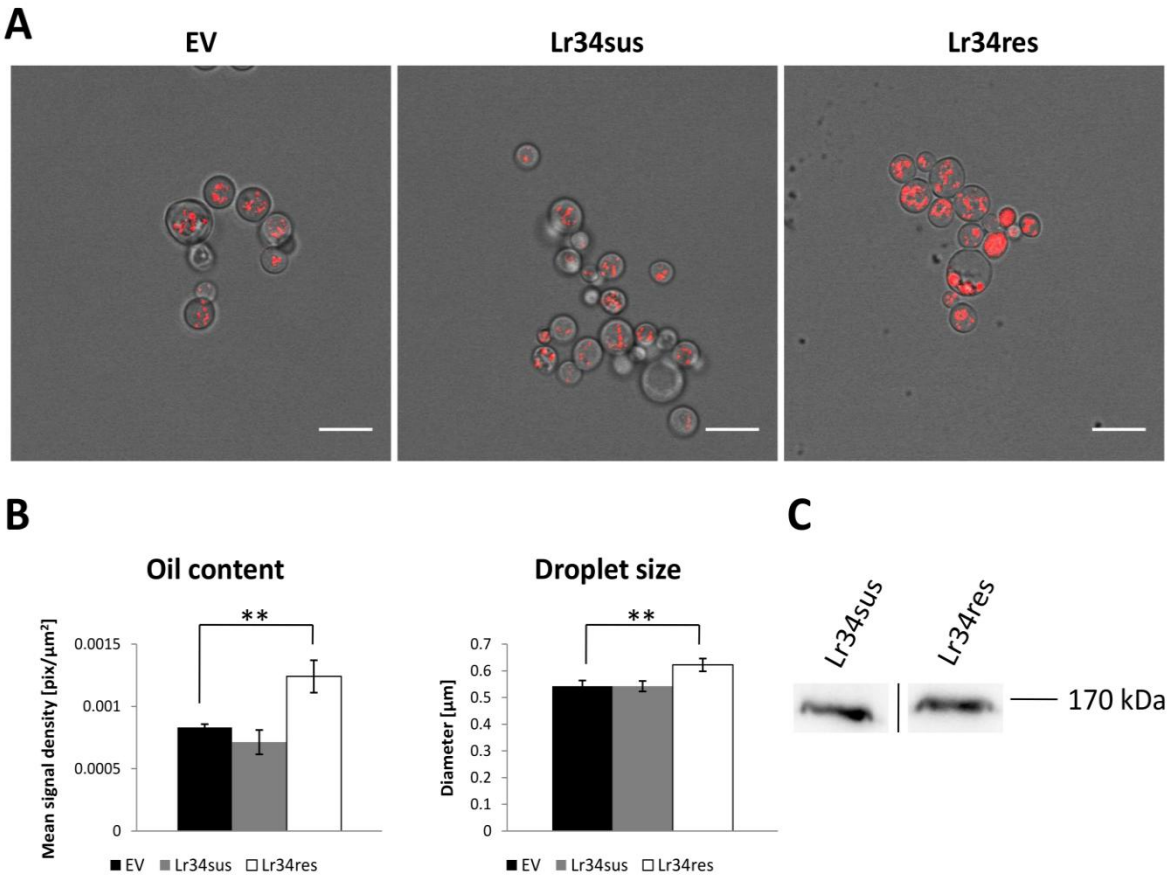

Figure 6 Oil body quantification in the *S. cerevisiae* Sec14^{ts} mutant CTY1-1A | (a) Yeast cells transformed with an empty vector or plasmids for the expression of Lr34sus and Lr34res, respectively, were grown to stationary phase and stained with Nile red for the microscopic visualization of oil bodies. Scale bars, 10 µm. (b) Determination of Nile red-derived fluorescence signal density as a measure of oil content and determination of average oil droplet size in transformed yeast cells. Values are means ± s.e.m. (n=6-10 for oil content, n=16-33 for droplet size). **P≤0.01, according to Student's t-test. (c) Protein levels of HA-tagged Lr34sus and Lr34res in CTY1-1A microsomal fractions.

2.4 Discussion

Resolving the molecular mechanism of disease resistance in cereals is of great importance, especially in view of the challenges in sustainable food supply in the future. The ABC transporter Lr34 from wheat is known to confer partial, durable and broad-spectrum disease resistance, which makes it a very interesting candidate for molecular investigations. It was demonstrated that the *Lr34res* gene can be functionally transferred into other grass species like rice, barley or maize, resulting in resistance against all tested species-specific biotrophic and hemibiotrophic pathogens, such as *Magnaporthe oryzae*, *Blumeria graminis* f. sp. *hordei* or *Exserohilum turcicum* (8-10). However, strong transgene expression can cause side-effects, such as triacylglycerol accumulation in barley leaves (11) or early senescence in barley and rice (8-9). The aim of this study was to characterize the activity of the ABC transporter Lr34, in an attempt to find a common cause for the observed diverse physiological effects.

Before investigating the activity of Lr34 *in planta*, the subcellular localization of the ABC transporter was elucidated. The absence of signaling peptides and the localization studies from related PDR-type transporters (17-19) suggested that Lr34 is most likely present in the PM. Fractionation of total membranes from transgenic BY2 cells indeed revealed that the HA-tagged Lr34res protein showed a signal correlation with the plasma membrane H^+-ATPase. Although it cannot be ruled out that Lr34res has additional subcellular destinations, this result confirmed that at least a major part of the expressed protein is present in the plant PM fraction.

Next, the transporter activity was determined. The lipid translocation assay in barley protoplasts suggested that Lr34res might be capable of internalizing PA, while exporting PS. Consequently, Lr34res on the one hand most likely acts as PS floppase and thereby reduces the amount of cytoplasmic PS and, on the other hand, might either directly flip PA from the exoplasmic to the cytoplasmic membrane leaflet or indirectly promote the accumulation of negatively charged PA to compensate for the reduced PS levels. The resulting elevation of the PA concentration at the inner PM leaflet by the activity of Lr34res could explain the upregulation of pathogenicity-related (PR) genes in transgenic barley (12) and further stress-responsive genes in *Lr34res* containing wheat varieties (13) through PA-mediated activation of the corresponding signaling pathways (14-16). Numerous reports have demonstrated that these cellular responses are highly dependent on the activity of specific phospholipases generating PA as signaling molecule (27-29). Moreover, the loss of PS in the cytoplasmic membrane leaflet could cause a detachment of intracellular proteins with PS-binding motifs from the PM and thereby alter their activity. For instance, the evolutionarily highly conserved copines contain calcium-dependent PS-binding domains and play important roles in

development and disease resistance, even though their precise molecular function is still unclear (30-32).

The investigation on the distribution of natural membrane lipids with specific PL-biosensors showed that a smaller portion of the expressed PS-biosensor (Lact-C2) is bound to the PM in *Lr34res*-expressing BY2 protoplasts compared to the control; instead, it accumulated in dot-like structures. Possibly, the fraction of Lr34res present in the Golgi and Golgi-derived vesicles on their way to the PM along the secretory pathway promotes PS translocation to the luminal membrane leaflet. Newly synthesized PS-binding biosensor molecules would therefore be able to bind these vesicles, explaining these intracellular dots. Interestingly, not only PS but also PI(4,5)P2 concentration was lowered in the cytoplasmic PM leaflet in *Lr34res*-expressing protoplasts, indicated by an enrichment of the corresponding biosensor in the cytosol and vacuoles. Reasons for this observation could either be a putative PI(4,5)P2 floppase activity of the transporter, or altered PI(4,5)P2 metabolism either through enhanced enzymatic conversion by phospholipase C (PLC) or through reduced biosynthesis by PI(4)P-kinase, since the activity of these two enzymes depends on the cytoplasmic PS and PA concentrations, respectively, of the PM (33, 34). Without further investigations on the PI(4,5)P2 content and distribution in the PM, none of these possibilities can be excluded. However, an enhanced PLC-mediated conversion of PI(4,5)P2 to DAG and IP_3 could explain several of the phenotypes associated with a strong expression of Lr34res in plant cells. For instance, PLC-derived DAG could be fed into TAG biosynthesis, which might explain the lipid accumulation profile in transgenic *Lr34res*-expressing barley plants (11). At the same time, the portion of DAG remaining intact in the membrane attracts C1 domain-containing proteins, whose activity is stimulated by increasing concentrations of this lipid intermediate. The most prominent example is protein kinase C (PKC), a universal regulator of signal transduction and cell proliferation in mammals that needs PS and DAG for its full activity (35). Interestingly, no PKC isoform has been found in plants but evidence exists that other protein kinases compensate for its absence. For instance, phosphoinositide-dependent kinase PDK1 mediates responses to ROS during root hair development and pathogen attack and its activity is stimulated by PA, the phosphorylated form of DAG (36).

Further support for a possible connection of Lr34res with TAG homeostasis can be gathered from the yeast experiments. When heterologously expressed in a *S. cerevisiae* mutant that is defective in PL transfer, Lr34res promoted the accumulation of neutral lipids in oil bodies. Oil body formation is not unique to yeast but occurs in almost all organisms and starts with the formation of microdomains at the endoplasmic reticulum (ER) and the local recruitment of lipid-biosynthesis enzymes. An increased production of neutral lipids, such as DAG, steryl-esters or TAG, at these sites promotes the extension and finally the conversion of these microdomains into oil bodies after their release from the ER (37). A defect in PL transfer in the $Sec14^{ts}$ mutant *per se* causes the formation of oil bodies, which hardly

accumulate in wild type yeast cells under normal growth conditions[1]. This observation could for example be explained by a reduction of PL delivery along the secretory pathway resulting in increased oil body formation for the storage of membrane lipid precursor molecules. Interestingly, the expression of Lr34res but not Lr34sus enhanced the density and size of oil bodies in this mutant. Because of similar protein expression levels it seems likely that a different activity of the two transporter versions is responsible for the increased oil body content and -size in *Lr34res*-expressing yeast cells. Presumably, Lr34res further impedes lipid transfer along the secretory pathway caused by its PL floppase activity and thereby enhances the phenotype of the $Sec14^{ts}$ mutant by promoting oil body formation. In analogy to the observations in *Lr34res*-expressing barley plants, the enhanced lipid content in the yeast mutant might also be derived from the indirect stimulation of PLC-mediated PL degradation caused by the activity of Lr34res assuming that this mechanism is identical in plants and in yeast.

When heterologously expressed in a *S. cerevisiae* wild type strain, Lr34sus and Lr34res but not the inactive transporter versions impaired growth, demonstrating that this effect is not caused by protein expression *per se* but by the activity of the transporters. On the one hand a strongly enhanced ATP consumption in yeast cells could reduce the availability of metabolic energy required for growth and on the other hand an Lr34-mediated redistribution of certain PLs within the PM might affect cell division as cell cycle regulation is known to be closely connected to lipid metabolism (38). Possibly, PL-specific toxins interfere with cell cycle regulation in a similar manner resulting in a loss of clearly distinguishable lag, exponential and stationary phases of growing yeast cells.

The functional analysis of Lr34 in yeast revealed that the tolerance to the toxic lyso-PC analogue miltefosine was neither influenced by Lr34sus nor Lr34res, meaning that this compound is not extruded by the ABC transporters. By contrast, the sensitivity to papuamide A, which specifically binds exposed PS, was increased by the expression of both Lr34 versions. Consequently, Lr34sus and Lr34res could work as PS floppases and would both be able to expose PS in the yeast PM supporting the data of the lipid translocation assay using barley protoplasts. Differential growth effects were also observed for the PE-binding toxin duramycin because the expression of both native Lr34 versions decreased yeast sensitivity to the drug, pointing to a reduction of the PE level in the exoplasmic PM leaflet. Interestingly, NBD-PE was not found to be a potential substrate of Lr34res in barley protoplasts, which might be due to the different membrane PL composition in yeast and barley that potentially influences the flipping rate of certain PLs so that PE is not a preferred substrate in barley compared to yeast. Another reason might be the difference in the fatty acid moieties of NBD-labelled PE compared to natural PE species, which potentially affects its interbilayer translocation.

In summary our results of the PL translocation assays in plants and in yeast indicate that Lr34res (and Lr34sus) work as PL floppases specific for PS and that the putative translocation of PA and PE to the inner leaflet of the PM is a secondary compensatory effect either due to increased spontaneous diffusion (PA) or due to stimulation of P-type ATPases that transfer PE and PS to the inner side of the PM. This hypothesis is supported by the fact that ABC transporters have so far not been described as bidirectional PL translocators but instead as unidirectional PL flippases or floppases with more or less narrow substrate spectra (3-6). Many of them – especially members of the G subfamily of ABC transporters – are associated with PS exposure in the PM in diverse organisms (4, 5) supporting the hypothesis that Lr34 has a similar function.

Despite the strong evidences that Lr34 is a translocator for certain PLs (especially PS), a detailed analysis of the substrate spectrum can only be performed in proteoliposomes with pure Lr34 protein that is reconstituted in liposomes of a defined lipid composition. In this system, differences between Lr34sus and Lr34res in the affinity for certain PLs could also be determined, which would finally allow a putative functional discrimination of the two transporter versions for the elucidation of the exact molecular mechanism behind the Lr34res-mediated pathogen resistance. Given that many ABCG transporters can transport very diverse compounds, this system would also allow to define if Lr34 might have further transport activities.

Nevertheless, this work shows that the wheat ABC transporter Lr34 changes the lipid environment in the PM by exposing PS on the outer membrane leaflet and by redistributing further PLs in a direct or indirect manner. The resulting membrane rearrangement could be a crucial factor for the pathogen resistance mechanism, the activation of stress responses, the accumulation of neutral lipids and the early induction of senescence in *Lr34res*-expressing plants.

2.5 Experimental Procedures

Material

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

DNA cloning and constructs

The genomic DNA of *Lr34* together with 35S promotor and N-terminal HA tag coding sequences was integrated into the p6U vector as described in ref. 8 in order to obtain constructs for *Agrobacterium*

tumefaciens-mediated transformation of BY2 cells (see below). The p6U vector contains an *hpt* cassette for hygromycine selection in target organisms. The phosphoinositide biosensor constructs P18R, P21R, P21Y and P24R were obtained from NASC (https://www.arabidopsis.org/portals/mutants/stockcenters.jsp) and were used directly for BY2 protoplast transfection. The PS biosensor Lact-C2 was created by PCR amplification of the C2 domain of lactadherin derived from the Lact-C2-GFP plasmid, which was a gift from Sergio Grinstein (Addgene plasmid # 22852, ref. 39), for cloning into the GATEWAY^{TM} (Invitrogen, Gaithersburg, MD, USA) compatible pDONR207 vector. Plant expression constructs were obtained by subcloning into the destination vector pUBC-RFP (40).

For expression in yeast, *Not*I-restricted *Lr34* cDNA including a C-terminal coding sequence for an HA tag was integrated into the pNEV-N vector (41) at the *Not*I restriction site to enable constitutive expression under the PMA1-promotor. Inactive Lr34 versions were generated by four consecutive PCR-based mutations of both Walker A and Walker B domains (see Table S1). All DNA constructs were verified by Sanger sequencing.

Plant material

Tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were grown in liquid LS-medium (Murashige-Skoog medium supplemented with 30 g/l sucrose, 0.37 g/l KH_2PO_4, 1 mg/l thiamine-HCl and 0.2 mg/l 2,4-dichlorophenoxyacetic acid) at 25°C under continuous dark conditions. For protein extraction and protoplast generation, cells were harvested 14 and 4 days, respectively, after sub-culturing.

Transgenic barley plants (lines BG9 and BG9 sib, ref. 8) were grown in standard soil under 12 h/d illumination (300 µmole m^{-2} s^{-1}) at 25°C.

BY2 cell culture and transformation

Suspension cultures of tobacco BY2 cells were maintained by weekly dilution (1:10) of cells into fresh LS-medium according to Nagata et al., 1992 (42) and cultured at 25°C with shaking at 130 rpm in the dark. The p6U vector containing *Lr34res* (genomic DNA) and N-terminal HA tag under the 35S promotor and the empty vector, respectively, were introduced by electroporation into the *A. tumefaciens* strain GV3101. Tobacco cells obtained from 50 ml of a 3-day-old culture were co-cultivated with 100 µl of an overnight culture of the transformed *A. tumefaciens strain* (adjusted to OD_{600}=1) on LS-plates supplemented with acetosyringone (6.6 mg/l) and hygromycin (30mg/l) for

selection. After 2 days cells were collected and washed three times with BY2 culture medium and were transferred to LS-plates supplemented with cefotaxime (200 mg/l) and hygromycin (30 mg/l). After 4-6 weeks transformed BY2 cells developed calli that were checked for protein presence and used as starting material for liquid cultures.

Protoplast isolation and transfection

Leaf mesophyll protoplasts were prepared from 8 day old transgenic (Lr34) and non-transgenic (sib) barley plants according to Kaiser et al., 1982 (43) for NBD-lipid uptake experiments.

Protoplasts of BY2 suspension cultured cells were prepared and transfected with phospholipid biosensor constructs as described in Miao & Jiang, 2007 (44).

NBD-lipid uptake in barley protoplasts

Dipalmitoyl-PC (DPPC) and fluorescence-labelled ((7-nitro-2-1,3-benzoxadiazol-4-yl)amino, NBD) Palmitoyl-(NBD-hexanoyl)-PS (NBD-PS), palmitoyl-(NBD-hexanoyl)-PE (NBD-PE), palmitoyl-(NBD-hexanoyl)-phosphocholine (NBD-PC), palmitoyl-(NBDhexanoyl)-phosphatidylglycerol (NBD-PG) and palmitoyl-(NBDhexanoyl)-phosphatidic acid (NBD-PA) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). NBD-lipid stocks were prepared as vesicles by sonication consisting of 60 mol% DPPC and 40 mol% NBD-lipid in buffer A (25 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.25 M sucrose). Leaf mesophyll protoplasts from transgenic and non-transgenic barley plants were normalized by adjusting their chlorophyll content to 200 µg/ml and incubated in W5 buffer (160 mM KCl, 125 mM $CaCl_2$, 5 mM glucose and 2 mM MES, pH 5.7) containing lipid vesicles to a final concentration of 2.5 µM (NBD-PC/NBD-PG), 5 µM (NBD-PA/NBD-PS) or 10 µM (NBD-PE) at 25°C and under mild agitation (300 rpm). Samples were taken at the indicated time points and protoplasts were washed twice by resuspending and centrifuging (100 g) in ice cold W5 buffer supplemented with 3% (w/v) bovine serum albumin to remove excessive lipids. All samples were boiled at 90°C for 30 s after adding hot isopropanol for heat inactivation of phospholipases. The phospholipid extraction was continued according to Bligh and Dyer (45) and the lipid-containing chloroform/methanol phase was collected, dried and resuspended in a small volume of chloroform before separation by thin-layer chromatography (TLC glass plates, prod-no: 100390, Merck) using chloroform/ethanol/water/trimethylamine (30/35/7/35, v/v/v/v; ref. 46). NBD-lipid standards were chromatographed on the same plate. Fluorescent lipid spots were visualized with an Imager (Fusion FX6) and signal intensities were quantified with ImageJ (http://imagej.nih.gov/ij/).

Membrane fractionation of BY2 microsomes

First, protoplasts were isolated from 14 day old transgenic *Lr34res*-expressing BY2 suspension cells according to Miao & Jiang, 2007 (44) in 2x cell vol enzyme solution for 2.5 h. The resulting protoplasts were collected by centrifugation (100 g, 6 min), resuspended in 1x cell vol ice cold buffer 1 (25 mM Tris-HCl pH 7.5, 5 mM EDTA, 1x protease inhibitor cocktail (PIC) (Roche, cat. No.: 11836170001)) and immediately homogenized with glass beads (1x cell vol) by vortexing (4 x 10 s, cooling in between). After removal of cell debris and glass beads (4000 rpm, 10 min, 6°C) the supernatant was transferred to ultracentrifugation tubes to obtain the total membrane fraction by centrifugation (100,000 g; 6°C; 45 min). The pellet was resuspended in STED 10 buffer (10% (w/v) sucrose, 10 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 0.2 mM DTT) and loaded on a linear sucrose gradient (10-50% sucrose in STED buffer). After ultracentrifugation (16 h; 4°C; 100,000 g) 18 fractions were harvested and their sucrose concentration measured by refractometry before western blotting.

Yeast strains and growth conditions

All growth assays were performed with the *S. cerevisiae* wt strain BY4741 (MATa his3 leu2 ura3 met15; EUROSCARF), while the *S. cerevisiae* mutant CTY1-1A (ura3 his3 lys2 sec14^{ts}, ref. 25) was used for investigations on oil body formation. Yeast cells were transformed by electroporation as described in Thompson et al., 1997 (47). Papuamide A (Flintbox, Lynsey Huxham; www.flintbox.com) and duramycin (Sigma-Aldrich, St. Louis, MO, USA) were prepared as 1 mg/ml and 2 mM stock solutions, respectively, dissolved in DMSO. Miltefosine (hexadecylphosphocholine; Calbiochem, La Jolla, CA, USA) was prepared as 30 mg/ml stock solution dissolved in ethanol. For toxicity assays in liquid medium overnight-grown (14-18 h) yeast cultures were diluted to OD_{600}=0.2 in selective SD medium (0.7% yeast nitrogen base, 2% glucose, 1x synthetic drop out media lacking uracil (Sigma-Aldrich, St. Louis, MO, USA)) and grown in 96 well plates at 30°C and shaking at 260 rpm. Appropriate volumes of DMSO and ethanol were added to control samples. All experiments were repeated independently at least four times. The growth inhibitory effects of the toxins were defined as the percentage of growth reduction under toxin application using the formula $(1-(OD_{600}tox(t_2)/OD_{600}tox(t_1))/ (OD_{600}con(t_2)/OD_{600}con(t_1)))*100$ with $OD_{600}tox(t_2)$ defined as the OD_{600} value at the end of the exponential growth phase with toxin application, while t_1 indicates the beginning of the exponential growth phase. The corresponding values were determined for the solvent controls (con).

Nile red staining of yeast cells

Cells were grown in selective SD medium for 16 h and harvested in the stationary phase. The OD_{600} was adjusted to 1.0 before cells were stained for 15 min with Nile Red (dissolved in DMSO, final concentration 5 µg/ml).

Yeast microsomal membrane preparation

Fresh yeast transformants were inoculated in selective SD medium and grown overnight at 30°C with 160 rpm shaking, before inoculation into SD medium at OD_{600}=0.2. After another 6-7 h incubation period under the same conditions, cells were harvested by centrifugation at 3,000 g at 4 °C for 5 min, washed in ice-cold water and resuspended 1/1 (w/v) in buffer 1 (see above). Cells were lysed by the addition of one volume ice-cold acid-washed glass beads (0.5 mm), followed by three cycles of 30 s homogenization with the fastprep® cell disruptor (MP Biomedicals, Santa Ana, USA) and 3 min rest on ice between cycles. Samples were centrifuged at 3,000 g at 4 °C for 10 min to eliminate glass beads and cell debris. After a washing step, supernatants were combined and total microsomal membrane fractions were collected by centrifugation (100,000 g; 4 °C; 45 min) and homogenized in buffer 2 (10 mM MES pH 7.8, 250 mM sucrose, 1x PIC).

Immunodetection

A rat monoclonal anti-HA-HRP coupled antibody (Roche, cat. No.: 12013819001, Anti-HA-Peroxidase, High Affinity, dil: 1:1000) was used for Lr34-HA detection. BY2 organelle marker proteins were visualized by the consecutive development with primary antibodies from Agrisera (Agrisera, Item No.: AS07 260-100, AS07 213, AS05 084A and AS08 325) according to the manufacturer's recommendations and secondary HRP-coupled goat anti-rabbit IgG antibody (Santa Cruz, Item No.: sc-2400).

All blots were developed with luminol and enhancer solution (WesternBright™ ECL HRP substrate, advansta) and signals were detected using a Fusion-FX6 imaging device.

Fluorescence microscopy and signal quantification

A Leica SP5 II spectral confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) was used for fluorescence microscopy. For visualization of plant tissues, a 40x/1.2 N.A. oil immersion objective was used and yeast imaging was done with a 63x/1.40 N.A. oil immersion objective. For Nile red visualization in yeast cells the dye was excited with an argon laser at 488 nm and emission

captured between 565-585 nm. Fluorescence signals were evaluated using the Leica software LAS X (version 1.1.0.12420). For the quantification of Nile red fluorescence as a measure of oil body content individual yeast cells were defined as regions of interest (ROIs) and the pixel intensity sum of the ROI stack was calculated and divided by the number of pictures to obtain the mean pixel intensity. For the determination of the mean oil body size the max oil body diameter in the stack was measured for each individual organelle in several yeast cells.

In plants, biosensor proteins with RFP were excited at 532 nm and emission spectra were recorded between 580 and 630 nm. The citrine-tagged version P21Y was excited at 488 nm and emission captured between 510-570 nm. Entire BY2 protoplasts were defined as ROI1 to obtain the total fluorescence signal. ROI2 as a measure of the intracellular signal was obtained by subtraction of the PM signal from ROI1. The dye FM4-64 was added to protoplasts at 17 µM for PM staining. FM4-64 was excited at 488 nm and emission was captured between 660-710 nm.

Statistical analyses

Statistical analyses for NBD-lipid uptake in barley protoplasts, biosensor quantification in BY2 protoplasts and oil body investigations in yeast were performed using a Student's t-test with a two-tailed distribution and two-sample unequal variance. The significance of growth differences in the yeast toxicity assay was evaluated using one-way ANOVA analysis and post-hoc t-tests (Bonferroni corrected).

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2.7 Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

2.8 Author contributions

JPD conducted all of the experiments, analyzed the results, and wrote the first version of the paper. RR assisted in the NBD-PL uptake experiment with barley protoplasts. RLLM, SH, EM and BK provided supervision and guidance at different project stages and helped improving the manuscript. All authors commented on the final version.

2.9 References

1. Higgins, C. F. (2001). ABC transporters: physiology, structure and mechanism–an overview. Research in microbiology, 152(3), 205-210.
2. Borst, P., & Elferink, R. O. (2002). Mammalian ABC transporters in health and disease. Annual Review of Biochemistry, 71(1), 537-592.
3. Decottignies, A., Grant, A. M., Nichols, J. W., de Wet, H., McIntosh, D. B., & Goffeau, A. (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. Journal of Biological Chemistry, 273(20), 12612-12622
4. Campos-Salinas, J., León-Guerrero, D., González-Rey, E., Delgado, M., Castanys, S., Pérez-Victoria, J. M., & Gamarro, F. (2013). LABCG2, a new ABC transporter implicated in phosphatidylserine exposure, is involved in the infectivity and pathogenicity of Leishmania. PLoS neglected tropical diseases, 7(4), e2179.
5. Woehlecke, H., Antje, P. O. H. L., Alder-Baerens, N., Hermann, L. A. G. E., & Herrmann, A. (2003). Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2). Biochemical Journal, 376(2), 489-495.
6. Krishnamurthy, S., Dixit, B. L., Gupta, C. M., Milewski, S., & Prasad, R. (2002). ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. Yeast, 19(4), 303-318.
7. Krattinger, S. G., Lagudah, E. S., Spielmeyer, W., Singh, R. P., Huerta-Espino, J., McFadden, H., ... & Keller, B. (2009). A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science, 323(5919), 1360-1363.
8. Risk, J. M., Selter, L. L., Chauhan, H., Krattinger, S. G., Kumlehn, J., Hensel, G., ... & Lagudah, E. S. (2013). The wheat Lr34 gene provides resistance against multiple fungal pathogens in barley. Plant biotechnology journal, 11(7), 847-854.

9. Krattinger, S. G., Sucher, J., Selter, L. L., Chauhan, H., Zhou, B., Tang, M., ... & Schaffrath, U. (2016). The wheat durable, multipathogen resistance gene Lr34 confers partial blast resistance in rice. Plant biotechnology journal, 14(5), 1261-1268.
10. Sucher, J., Boni, R., Yang, P., Rogowsky, P., Büchner, H., Kastner, C., ... & Keller, B. (2017). The durable wheat disease resistance gene Lr34 confers common rust and northern corn leaf blight resistance in maize. Plant biotechnology journal, 15(4), 489-496.
11. Bucher, R. R. (2017). Mass-spectrometry Based Metabolomic Approaches to Characterize Plant Pathogen Resistance in Cereals and the Flavonols Modifying Development in *Arabidopsis Thaliana* (Doctoral dissertation, Universität Zürich).
12. Chauhan, H., Boni, R., Bucher, R., Kuhn, B., Buchmann, G., Sucher, J., ... & Glauser, G. (2015). The wheat resistance gene *Lr34* results in the constitutive induction of multiple defense pathways in transgenic barley. The Plant Journal, 84(1), 202-215.
13. Hulbert, S. H., Bai, J., Fellers, J. P., Pacheco, M. G., & Bowden, R. L. (2007). Gene expression patterns in near isogenic lines for wheat rust resistance gene *Lr34/Yr18*. Phytopathology, 97(9), 1083-1093.
14. Xue, H., Chen, X., & Li, G. (2007). Involvement of phospholipid signaling in plant growth and hormone effects. Current opinion in plant biology, 10(5), 483-489.
15. Mishra, G., Zhang, W., Deng, F., Zhao, J., & Wang, X. (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. Science, 312(5771), 264-266.
16. Zhao, J. (2015). Phospholipase D and phosphatidic acid in plant defence response: from protein–protein and lipid–protein interactions to hormone signalling. Journal of Experimental Botany, 66(7), 1721-1736.
17. Kang, J., Hwang, J. U., Lee, M., Kim, Y. Y., Assmann, S. M., Martinoia, E., & Lee, Y. (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proceedings of the National Academy of sciences, 107(5), 2355-2360.
18. Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., & Maeshima, M. (2006). Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. Plant and Cell Physiology, 47(3), 309-318.
19. Crouzet, J., Roland, J., Peeters, E., Trombik, T., Ducos, E., Nader, J., & Boutry, M. (2013). NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. Plant molecular biology, 82(1-2), 181-192.
20. Simon, M. L. A., Platre, M. P., Assil, S., Wijk, R., Chen, W. Y., Chory, J., ... & Jaillais, Y. (2014). A multi-colour/multi-affinity marker set to visualize phosphoinositide dynamics in *Arabidopsis*. The Plant Journal, 77(2), 322-337.

21. Märki, F., Hänni, E., Fredenhagen, A., & van Oostrum, J. (1991). Mode of action of the lanthionine-containing peptide antibiotics duramycin, duramycin B and C, and cinnamycin as indirect inhibitors of phospholipase A2. Biochemical pharmacology, 42(10), 2027-2035.
22. Hanson, P. K., Malone, L., Birchmore, J. L., & Nichols, J. W. (2003). Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. Journal of Biological Chemistry, 278(38), 36041-36050.
23. Andjelic, C. D., Planelles, V., & Barrows, L. R. (2008). Characterizing the anti-HIV activity of papuamide A. Marine drugs, 6(4), 528-549.
24. Parsons, A. B., Lopez, A., Givoni, I. E., Williams, D. E., Gray, C. A., Porter, J., ... & Wang, J. (2006). Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. Cell, 126(3), 611-625.
25. Bankaitis, V. A., Malehorn, D. E., Emr, S. D., & Greene, R. (1989). The *Saccharomyces cerevisiae SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. The Journal of Cell Biology, 108(4), 1271-1281.
26. Chen, W., Zhang, C., Song, L., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. Journal of microbiological methods, 77(1), 41-47.
27. De Jong, C. F., Laxalt, A. M., Bargmann, B. O., De Wit, P. J., Joosten, M. H., & Munnik, T. (2004). Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. The Plant Journal, 39(1), 1-12.
28. Wang, X. (2005). Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. Plant Physiology, 139(2), 566-573.
29. Testerink, C., & Munnik, T. (2011). Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. Journal of experimental botany, 62(7), 2349-2361.
30. Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J., & Cohen, J. (1998). The copines, a novel class of C2 domain-containing, calciumdependent, phospholipid-binding proteins conserved from *Paramecium* to humans. Journal of Biological Chemistry, 273(3), 1393-1402.
31. Zou, B., Hong, X., Ding, Y., Wang, X., Liu, H., & Hua, J. (2016). Identification and analysis of copine/BONZAI proteins among evolutionarily diverse plant species. Genome, 59(8), 565-573.
32. Jambunathan, N., Siani, J. M., & McNellis, T. W. (2001). A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. The Plant Cell, 13(10), 2225-2240.

33. Ananthanarayanan, B., Das, S., Rhee, S. G., Murray, D., & Cho, W. (2002). Membrane targeting of C2 domains of phospholipase C-δ isoforms. Journal of Biological Chemistry, 277(5), 3568-3575.
34. Moritz, A., De Graan, P. N., Gispen, W. H., & Wirtz, K. W. (1992). Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. Journal of Biological Chemistry, 267(11), 7207-7210.
35. Johnson, J. E., Giorgione, J., & Newton, A. C. (2000). The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. Biochemistry, 39(37), 11360-11369.
36. Anthony, R. G., Henriques, R., Helfer, A., Meszaros, T., Rios, G., Testerink, C., ... & Bögre, L. (2004). A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. The EMBO journal, 23(3), 572-581.
37. Murphy, D. J., & Vance, J. (1999). Mechanisms of lipid-body formation. Trends in biochemical sciences, 24(3), 109-115.
38. Jackowski, S. (1994). Coordination of membrane phospholipid synthesis with the cell cycle. Journal of Biological Chemistry, 269(5), 3858-3867.
39. Yeung, T., Gilbert, G. E., Shi, J., Silvius, J., Kapus, A., & Grinstein, S. (2008). Membrane phosphatidylserine regulates surface charge and protein localization. Science, 319(5860), 210-213.
40. Grefen, C., Donald, N., Hashimoto, K., Kudla, J., Schumacher, K., & Blatt, M. R. (2010). A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. The Plant Journal, 64(2), 355-365.
41. Sauer, N., & Stolz, J. (1994). SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. The Plant Journal, 6(1), 67-77.
42. Nagata, T., Nemoto, Y., & Hasezawa, S. (1992). Tobacco BY-2 cell line as the “HeLa” cell in the cell biology of higher plants. International Review of Cytology, 132, 1-30.
43. Kaiser, W. M. (1982). Correlation between changes in photosynthetic activity and changes in total protoplast volume in leaf tissue from hygro-, meso-and xerophytes under osmotic stress. Planta, 154(6), 538-545.
44. Miao, Y., & Jiang, L. (2007). Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nature protocols, 2(10), 2348.
45. Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology, 37(8), 911-917.

46. Weingärtner, A., Kemmer, G., Müller, F. D., Zampieri, R. A., dos Santos, M. G., Schiller, J., & Pomorski, T. G. (2012). *Leishmania* promastigotes lack phosphatidylserine but bind annexin V upon permeabilization or miltefosine treatment. PloS one, 7(8), e42070.
47. Thompson, J. R., Register, E., Curotto, J., Kurtz, M., & Kelly, R. (1998). An improved protocol for the preparation of yeast cells for transformation by electroporation. Yeast, 14(6), 565-571.

2.10 Footnotes

This work was supported by funding from the University of Zürich and ERC Advanced Grant Durable resistance (249996).

The abbreviations used are: PL, phospholipid; PM, plasma membrane; ABC, ATP-binding cassette; PDR, pleiotropic drug resistance; PE, phosphatidylethanolamine; PS, phosphatidylserine; TAG, triacylglycerol; PR, pathogenicity related; PLase, phospholipase; PA, phosphatidic acid; DAG, diacylglycerol; IP_3, inositol trisphosphate; PLD, phospholipase D; ABA, abscisic acid; ER, endoplasmic reticulum; HA, human influenza hemagglutinin; BY2, bright yellow 2; HRP, horse radish peroxidase; NBD, 7-nitro-2-1,3-benzoxadiazol-4-yl; PC, phosphatidylcholine; PG, phosphatidylglycerol; PIP, phosphatidylinositolphosphate; PLC, phospholipase C; PKC, protein kinase C; PDK, phosphoinositide-dependent kinase; ROS, reactive oxygen species; RFP, red fluorescent protein

[1]J. Deppe, unpublished observations

2.11 Supporting information

Table S1 Mutagenesis sites for generation of inactive Lr34 versions

Motif (position in Lr34r)	Original sequence	Mutated sequence
Walker A1 (168-175)	GPPGCGKS	GPPGCAAS
Walker B1 (327-332)	AYFMDE	AYFMAE
Walker A2 (846-853)	GVSGAGKT	GVSGAAAT
Walker B2 (973-978)	IILMDE	IILMAE

Table S2 Primers used in this study

Primer	Sequence (5'-3')	Experiment	Reference
Lact-C2-attB1_fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGTGCACTGAACCCCTAGGCCTG	PS-biosensor	This work
Lact-C2-attB2_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCACAGCCCAGCAGCTCCAC	PS-biosensor	This work
Lr34-Not1_fwd	GCGGCCGCATGGAGGGCCTCGCAAGAGA	Lr34 expression in yeast	This work
Lr34-Not1_rev	GCGGCCGCTTACCTCTTCTGGAAATTAAGTTTCT	Lr34 expression in yeast	This work
Muta-WA1(Lr34)_fwd	CTGGATGTGCCGCTAGCACTCTGTTGCGAGCT	Inactive Lr34 versions	This work
Muta-WA1(Lr34)_rev	GAGGTCCCAGTAGAAGAGTCAATCTGCAGGGTTTG	Inactive Lr34 versions	This work
Muta-WA2(Lr34)_fwd	GAGCTGCCGCCACAACTCTACTAGATGTATTAGCAG	Inactive Lr34 versions	This work
Muta-WA2(Lr34)_rev	CACTAACACCCATTAGTGCAGAAAGAACACCG	Inactive Lr34 versions	This work
Muta-WB1(Lr34)_fwd	CATACTTTATGGCTGAAATATCAAATGGTCTGGATAG	Inactive Lr34 versions	This work
Muta-WB1(Lr34)_rev	CACTTGCGGGGCCCACAATCATCTCGGCTGTG	Inactive Lr34 versions	This work
Muta-WB2(Lr34)_fwd	CAACAACAGGTTTAGATACAAGG	Inactive Lr34 versions	This work
Muta-WB2(Lr34)_rev	GTTCAGCCATTAGTATGATTGATGG	Inactive Lr34 versions	This work

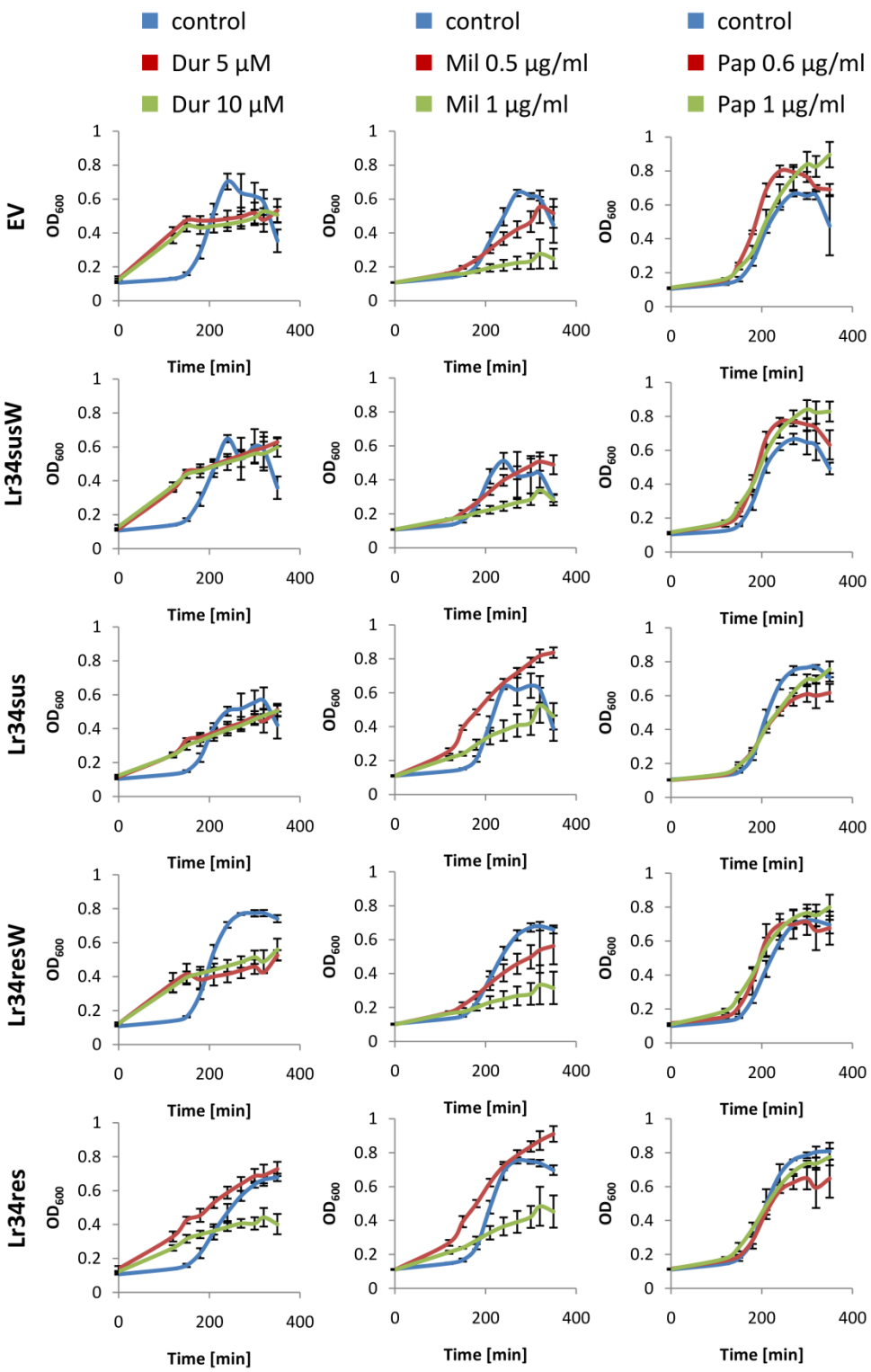

Figure S1 Growth curves of yeast BY4741 cells expressing different Lr34 versions in the presence of PL-specific toxins | The growth pattern of yeast cells containing an empty vector (EV) or expressing inactive Lr34sus (Lr34susW) or Lr34res (Lr34resW) or the active protein versions Lr34sus or Lr34res was monitored. Cells were grown in media containing either the cytotoxic drugs duramycin (Dur), miltefosine (Mil) or papuamide A (Pap) at the indicated concentrations or DMSO (Dur, Pap) or ethanol (Mil) at the corresponding concentrations as controls. OD_{600} was measured at the indicated time points. Values are means ± s.e.m. (n=4-6).

3 Determination of PL-degrading activity and visualization of incorporated NBD-PA in transgenic *Lr34res*-expressing barley protoplasts

3.1 Introduction

Often the activation of stress and hormone responses in plants is mediated by phospholipases (PLases) of various classes. These enzymes cleave membrane PLs at different intramolecular sites (Fig. 3.1) and thereby generate signaling compounds that activate or deactivate downstream factors, *e.g.* protein kinases and phosphatases, resulting in stress-responsive gene expression (Munnik *et al*., 1998). Phospholipase A (PLA) constitutes one class of PLases and removes fatty acid (FA) moieties either at the sn1-position (PLA1), the sn2-position (PLA2) or without stereoselectivity at one of these two positions (PLB) yielding lyso-PLs remaining in the membrane and free FAs (Dennis, 2015). The latter constitute precursors for the biosynthesis of hormones, such as prostaglandins in humans or jasmonic acid in plants (Irvine, 1982; Dave & Graham, 2012). In contrast, phospholipase C (PLC) cleaves off the phosphorylated head group of PLs resulting in the formation of diacylglycerol (DAG) in the membrane. This intermediate either directly serves as signaling molecule by attracting C1-domain containing proteins to the membrane or is further phosphorylated to phosphatidic acid (PA), the most important signaling lipid in plants (Munnik, 2001). Most PLC isoforms are specific for phosphoinositides and play important roles in the IP_3-mediated release of Ca^{2+} from intracellular stores. Finally, phospholipase D (PLD), the third class of PLases, removes the non-phosphorylated head group from PLs and thereby directly produces PA, the most important lipid signaling compound in plants. For example, PA is a crucial factor in the activation of ABA responses for guard cell movement because it promotes membrane sequestration of the protein phosphatase ABI1 (ABA-insensitive 1), an inhibitor of the ABA signal transduction cascade (Zhang *et al*., 2004). In addition, strong PA accumulation was observed during pathogen interactions, which highlights its role in biotic stress responses (Zhao, 2015).

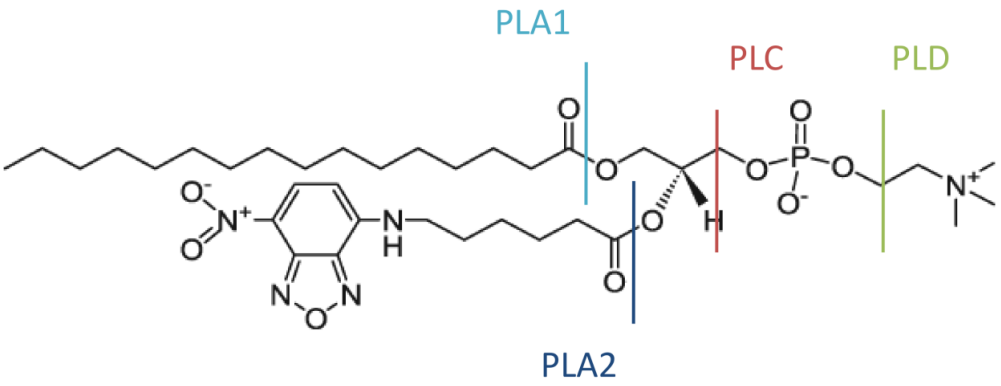

Figure 2.1 Cleavage sites of PLases depicted for 16:0-06:0 NBD-PC | PLA1 and PLA2 hydrolyze the ester bonds between the glycerol backbone and FA moieties yielding free FAs (palmitic acid and hexanoyl-NBD) and the corresponding lyso-PC species. PLC removes the phosphorylated head group (phosphocholine) from the PL sparing 16:0-06:0 NBD-DAG, while PLD cleaves before the phosphate group and thereby generates 16:0-06:0 PA and choline.

When fluorescence-labelled (NBD)-PLs are taken up by cells they usually do not remain in the plasma membrane (PM) but are transported to other cellular destinations where they can accumulate and further be metabolized. As the activity of several PL-modifying enzymes is altered during cellular stress responses, which are also activated in *Lr34res*-expressing plants, it was investigated in this chapter if the activity of the ABC transporter influences the turnover of specific NBD-PLs. Moreover, Lr34res was found to promote the accumulation of NBD-PA in barley protoplasts (see chapter 2). Therefore, fluorescence microscopy was used in a follow-up experiment to identify the cellular destinations of the incorporated NBD-PA and potentially link this result to the observed accumulation of neutral lipids (Bucher, 2017) as a putative consequence of enhanced PA to TAG conversion.

3.2 Results

In order to check if PLase activity in transgenic barley is altered by Lr34res, leaf mesophyll protoplasts of transgenic *Lr34res*-expressing (BG9; Risk *et al*., 2013) and control plants (sib) were incubated for 16 hours with NBD-labelled PLs, which can be recognized as substrates by all PLases in spite of the attached fluorophore at the sn2-fatty acid moiety (Poulsen *et al*., 2015). After PL extraction and thin layer chromatography the signals derived from degraded NBD-PLs were quantified and related to the total amount of ingested lipid (Fig. 3.2). As a measure of PLD and PLC-DAG-kinase activity, the generated PA levels were determined. While PC and PE were converted at similar rates to PA (7-8 % and 4-5 %, respectively) by sib and BG9 protoplasts, significantly less PS was degraded to PA in the latter (26 % to 36 %). Interestingly, in general a much higher proportion of the ingested PS was converted to PA compared to PC and PE. In addition, DAG formation was analyzed in order to characterize PL degradation by PLC and (PLD)-PA phosphatase. Obviously, the expression of *Lr34res* had no influence on the conversion rate of PA and PC to DAG meaning that these enzymes were not affected in their activity in the presence of the ABC transporter. However, considering that only a few PLs could be tested in this assay, it cannot be excluded that other PLs (especially PI(4,5)P2) are preferentially catabolized by PLC when Lr34res is active. Lastly, the PE-derived NBD-containing free FAs were quantified for the determination of PLA2 activity. In general, only a small amount of the ingested PE was metabolized by PLA2 (2-2.5 %) and all other tested PLs showed even smaller free FA levels, which could not be quantified. PLA2 activity was similar in sib and BG9 protoplasts meaning that the generation of free FAs from PE is not altered by Lr34res. In conclusion, Lr34res does not affect the degradation rate of PA, PC and PE by PLases. Only the conversion of PS to PA was significantly reduced in the presence of the ABC transporter.

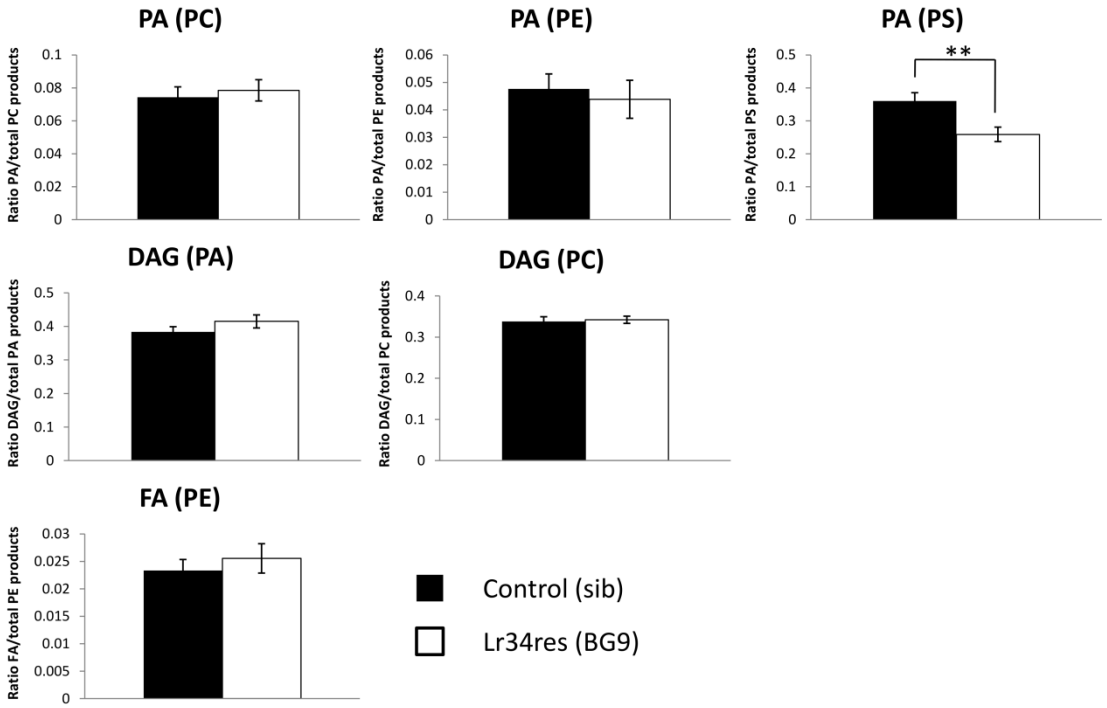

Figure 3.2 Determination of PLase activity in barley protoplasts | Leaf mesophyll protoplasts were isolated from *Lr34res*-expressing (BG9) and the corresponding non-transgenic (sib) barley plants and incubated for 16 h with NBD-phosphatidic acid (PA), NBD-phosphatidylethanolamine (PE), NBD-phosphatidylcholine (PC) or NBD-phosphatidylserine (PS) (indicated in brackets). Afterwards PLs were extracted and the fluorescence of the corresponding NBD-PLs and their degradation products NBD-phosphatidic acid (PA), NBD-diacylglycerol (DAG) and NBD-hexanol (FA) quantified. Values are means ± s.e.m. (n=3), **P≤0.01, according to Student's t-test.

To address if the incorporated NBD-PA simply accumulates in the PM or if it is transported to other cellular destinations (for further metabolization), *Lr34res*-expressing barley protoplasts were incubated overnight to enable a complete processing of the incorporated lipids. Afterwards, the final destination of NBD-PA within the protoplasts was investigated by fluorescence microscopy. NBD-PA did in fact not remain in the PM but instead accumulated in dot-like structures around chloroplasts within the cytosol (Fig. 3.3). To clarify the identity of these organelles, protoplasts were stained with Nile red, which is only fluorescent when embedded in a hydrophobic environment, typically found in oil bodies. When comparing the NBD and Nile red fluorescence, the overlap between both signals in the dot-like structures became apparent. However, another part of the NBD fluorescence was diffusely distributed around plastids at the margin of the protoplast. In conclusion, the incorporated (and metabolized) NBD-PA did not remain in the PM but instead accumulated in organelles that can be stained with Nile red and in addition in a cellular lumen surrounding plastids.

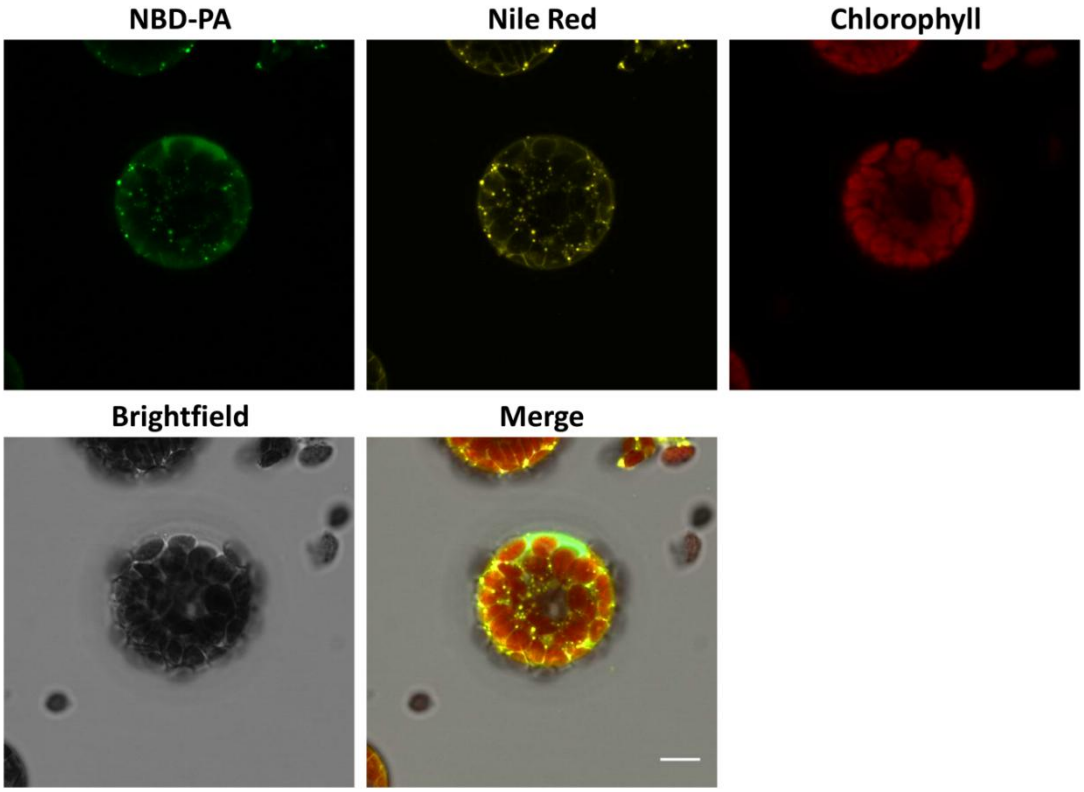

Figure 3.3 Microscopic analysis of NBD-PA accumulation in barley protoplasts | Protoplasts were isolated from first leaves of barley seedlings (line BG9) and incubated with NBD-PA for 16 h for the microscopic analysis of incorporated and metabolized PLs. As a marker for oil bodies Nile red was added to protoplasts. The third channel shows chlorophyll fluorescence. Scale bar, 10 µm.

3.3 Discussion

Physiological stress responses are often associated with large cellular reorganization processes including modifications of the PM by PLases (Spiegel *et al*., 1996; Munnik, 1998; Zhao, 2015). These enzymes degrade structural PLs with consequences for the physical membrane characteristics and further for the membrane-bound proteome. Moreover, the signaling molecules generated by these enzymes, *e.g.* PA or IP_3, evoke various cellular responses, such as the activation of signal transduction cascades or the release of Ca^{2+} from internal stores (Hanson *et al.*, 2004; Zhang *et al*., 2004). The observation that several stress-responsive genes are activated in transgenic *Lr34res*-expressing plants (Chauhan *et al.*, 2015; S. Krattinger, personal communication) led to the assumption that the ABC transporter might manipulate PL metabolism not only by redistributing certain PLs within the membrane but also by modulating PLase activity in an indirect manner. Because on the one hand the access to specific PLs for degradation might be facilitated by their exposure towards the cytosol and

on the other hand, an altered electrostatic field on the cytoplasmic PM side might attract PLases and thereby stimulate their activity (Rebecchi et al., 1992; Murray & Honig, 2002). However, at least the conversion rate of PA, PC and PE was not significantly changed meaning that enzymes recognizing these PLs as substrates, such as PA phosphatases, non-PI-specific PLCs or PLDs, were not significantly affected in their activity through Lr34res. Considering that the activity of these PLases is not dependent on the cytoplasmic PS level (which is the major factor affected by Lr34res) this result is however not surprising. Nevertheless, the PLase assay clearly demonstrated that Lr34res decreased the conversion rate of PS to PA. The most convincing explanation is that PS is translocated to the exoplasmic membrane leaflet by Lr34res and is therefore less accessible for degradation by PLases (especially PLDs). Alternatively, this result could be explained by the reduced cytoplasmic PI(4,5)P2 level in *Lr34res*-expressing protoplasts because PLDβ and PLDγ are known to require this phosphoinositide for their PS-hydrolyzing activity (Pappan *et al*., 1998).

Another observation was that a big portion of the ingested PA was independently on Lr34res expression converted to DAG due to PA phosphatase activity. In this case, it must be considered that PA degradation happens very fast (unpublished observation) so that putative differences between *Lr34res*-expressing and control protoplasts can obviously not be determined at the endpoint after 16 h incubation time. Consequently, short incubation times using high concentrations of NBD-PA might reveal an enhanced lipid turnover in the presence of Lr34res. Taking into account that the ABC transporter promotes PA accumulation in barley protoplasts (see chapter 2.3.2), it could be assumed that more PA is used for conversion into neutral lipids that are finally stored in oil bodies because PA dephosphorylation by PA phosphatase is the key step in glycerolipid biosynthesis (Athenstaedt & Daum, 1999).

Unfortunately, several interesting PL candidates, such as the PIPs, could not be tested for altered conversion rates in this assay. For example, the activity of PI-specific PLCs is modulated by PS (Lomasney *et al*., 1999) so that an enhanced removal of PS from the cytoplasmic membrane leaflet by Lr34res would likely affect the turnover of this PL class by PLC. This would further be in agreement with the biosensor studies demonstrating a reduction of cytosolically exposed PI(4,5)P2 in the PM of *Lr34res*-expressing BY2 protoplasts (see chapter 2.3.3). It must also be considered that part of the PLA-generated free FAs might get lost during the lipid extraction procedure because of their reduced hydrophobicity. This might be the reason why PLA activity could not be determined for PA, PC and PS assuming that these PLs are recognized as substrates.

In conclusion, the activation of stress responses in *Lr34res*-expressing plants can obviously not be traced back to an increased PLase-mediated turnover of PA, PC or PE to signaling molecules, such as

DAG or PA. Nevertheless, Lr34res might very well influence the activity of PI(4,5)P2-specific PLC leading to DAG and IP_3 production and – as a further step – the liberation of Ca^{2+} from internal stores, which might be the trigger for the activation of intracellular signal transduction cascades. Moreover, it cannot be excluded that Lr34res stimulates the activity of PLA and thereby increases the cellular levels of free FAs and lyso-PLs. To avoid the severe toxic effects of accumulating free FAs and to maintain membrane lipid homeostasis, the liberated FAs could for example be stored as TAG in oil bodies (as proposed for yeast; Petschnigg *et al.*, 2009) or be imported in their activated form into mitochondria and peroxisomes, respectively, for degradation (Goepfert & Poirier, 2007). A high PLA activity would in addition affect the physical properties of the PM because the accumulation of lyso-PLs severely affects the membrane order with impact on several membrane-membrane and membrane-protein interactions (Mishima *et al*., 2004).

In the next experiment fluorescence microscopy was applied to track the cellular distribution of incorporated (and metabolized) NBD-PA. While part of the fluorescent lipids accumulated in dot-like structures, another portion was diffusely distributed around plastids at the margin of the protoplast, most likely constituting the cytosol. The dot-like structures could be identified as oil bodies because their fluorescence overlapped with the oil body-specific stain Nile red. Consequently, NBD-PA is either directly used for the formation of the PL monolayer surrounding these organelles or it is converted to NBD-DAG by PA phosphatase (and potentially further acylated to NBD-TAG) and then stored as neutral lipid within these organelles. Although oil bodies are derived from microdomains of the ER (Kohlwein *et al.*, 2013), this result clearly shows that they may be extended by incorporating lipids from other cellular sources, such as the PM. This mechanism might explain the enhanced TAG content in *Lr34res*-expressing barley leaves (Bucher, 2017) as a consequence of Lr34res-promoted PA accumulation and its consecutive conversion to neutral lipids leading to oil body extension. Interestingly, another part of the NBD-PA derived fluorescence could be located to the cytosol. Presumably, the fluorophore-containing fatty acid moiety is cleaved off by phospholipase A (PLA2) and then diffuses from the membrane to the cytosol because of the loss in hydrophobicity. These observations already indicate that NBD-PLs are indeed not just incorporated into the PM of the plant cell as structural components but are recognized as substrates for PL-metabolizing enzymes that can convert NBD-PLs into various intermediates. A reduction of PA levels in the PM seems especially plausible as this lipid acts as a highly potent signaling molecule (Munnik, 2001), which would completely disturb the cellular physiology. Obviously, plant cells overcome this problem on the one hand by sequestering PA in oil bodies, where it is potentially fed into the biosynthesis of neutral lipids, and on the other hand by cleaving off one fatty acid moiety to generate free FAs and lyso-PA that can further be degraded to non-physiologically active compounds.

3.4 Materials and Methods

Material

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) when not stated differently.

Plant material

Transgenic barley plants (lines BG9 and BG9 sib; Risk *et al*., 2013) were grown in standard soil under 12 h/d illumination.

Protoplast isolation

Leaf mesophyll protoplasts were prepared from 8 day old *Lr34res*-expressing barley plants (line BG9) according to Kaiser *et al*., 1982. Protoplast density was adjusted to 200 µg/ml total chlorophyll content before each experiment (Arnon, 1949).

Protoplast staining and fluorescence microscopy

Barley protoplasts were incubated with 2.5 µM NBD-PA for 16 h in the dark. Immediately before microscopy the lipophilic dye Nile red was added (dissolved in DMSO, final concentration 5 µg/ml). A Leica SP5 II spectral confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) was used for fluorescence microscopy. Stained protoplasts were visualized with a 40x/1.2 N.A. oil immersion objective. NBD-PA and Nile red were excited at 458 and 488 nm and the emission captured between 500-560 nm and 560-620 nm, respectively, while chlorophyll fluorescence was captured between 719-750 nm.

NBD-lipid uptake in barley protoplasts for determination of PL activity

Dipalmitoyl-PC (DPPC), palmitoyl-(NBD-hexanoyl)-PS (NBD-PS), palmitoyl-(NBD-hexanoyl)-PE (NBD-PE), palmitoyl-(NBD-hexanoyl)-phosphocholine (NBD-PC) and palmitoyl-(NBDhexanoyl)-phosphatidic

acid (NBD-PA) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). NBD-lipid stocks were prepared as vesicles by sonication consisting of 60 mol% DPPC and 40 mol% NBD-lipid in buffer A (25 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.25 M sucrose). Leaf mesophyll protoplasts from transgenic and non-transgenic barley plants were normalized to their chlorophyll content (see above) and incubated in W5 buffer (160 mM KCl, 125 mM $CaCl_2$, 5 mM glucose and 2 mM MES, pH 5.7) containing lipid vesicles to a final concentration of 2.5 µM NBD-PL at 25°C and under mild agitation (300 rpm) with lipid vesicles at 25°C. Samples were taken after 16 h and washed twice in ice cold W5 buffer supplemented with 3% (w/v) bovine serum albumin to remove non-incorporated lipids. All samples were boiled at 90°C for 30 s after adding hot isopropanol for heat inactivation of phospholipases. The phospholipid extraction was continued according to Bligh & Dyer, 1959 and the lipid containing chloroform/methanol phase was collected, dried and resuspended in a small volume of chloroform before separation by thin-layer chromatography (TLC glass plates, prod-no: 100390, Merck) using chloroform/ethanol/water/trimethylamine (30/35/7/35, v/v/v/v; Weingärtner *et al*., 2012). NBD-lipid standards were chromatographed on the same plate. Fluorescent lipid spots were visualized using a Fusion FX6 imaging device and signal intensities were quantified using ImageJ (http://imagej.nih.gov/ij/).

4 Purification of Lr34 for reconstitution into liposomes for functional studies

4.1 Introduction

Despite the clear indications that Lr34 works a translocator for certain PLs (especially PS), a detailed analysis of the activity and substrate spectrum of the ABC transporter as well as the determination of putative differences in substrate preference between Lr34sus and Lr34res can only be performed with proteoliposomes containing pure Lr34 protein reconstituted in liposomes of a defined lipid composition. As a first step, it is essential to produce large protein amounts containing a tag for affinity purification. Typically yeast cells (*Saccharomyces cerevisiae* and *Pichia pastoris*) or insect cells (derived from *Spodoptera frugiperda*, *e.g.* Sf9) are the preferred systems for large-scale expression of ABC transporters (Conseil *et al*., 2000; Özvegy *et al*., 2001; Cai & Gros, 200) that usually require complex post-translational modifications and chaperone-mediated folding. Therefore, *S. cerevisiae* was chosen as the first expression system because the Lr34 protein could already be detected in the

yeast microsomal fraction (see chapter 2.3.4). In addition, the ABC transporter was also produced in tobacco BY2 suspension cells that might be more suitable for high expression levels of a wheat protein due to a more similar codon usage of this plant species compared to yeast.

4.2 Results

On the one hand His-tagged Lr34 protein was produced in the *S. cerevisiae* wild type strain BY4741 and on the other hand the HA-tagged version was produced in tobacco BY2 cells because both systems have been proven to be suitable for the expression of the ABC transporter. In case of the stably transformed BY2 cells the lines with the strongest Lr34 signals were selected for protein production. Of the four Lr34sus lines, only L s2 and L s3 showed a signal, while three Lr34res lines (L r5, L r7 and L r13) were tested positive (Fig. 4.1). The two lower bands in most of the fractions indicate that part of the protein was already degraded. Nevertheless, the relative quantification allowed the selection of L s2 and L r5 as the best candidates for further investigations.

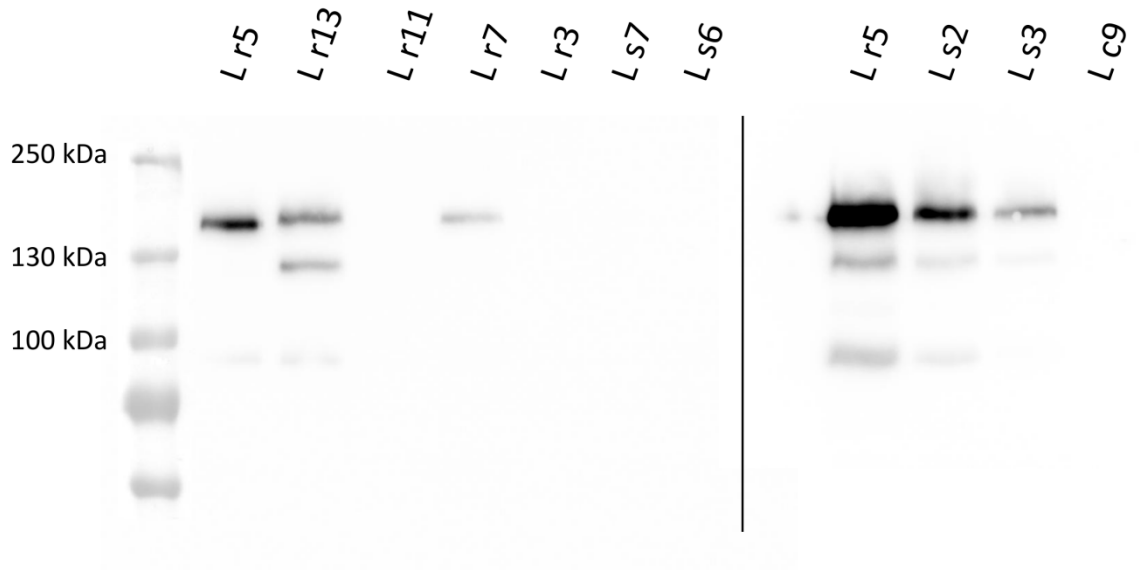

Figure 4.1 Western blot of microsomal fractions from different Lr34 expressing BY2 lines | Microsomal fractions from four individual BY2 lines of Lr34sus (L s2-s7) and five of Lr34res (L r3-13) were isolated and checked for the presence of the HA-tagged Lr34 protein by western blotting. BY2 cells only expressing the selection marker (L c9) were used as negative control.

For the expression in yeast, cells were harvested at different culture densities in order to maximize Lr34 protein production. In addition, the closely related ABC transporter Pdr5 from *S. cerevisiae* was chosen as control for all consecutive experiments. Apparently, the ratio of target protein to total protein was higher for cells below OD_{600}=2 compared to cells in the late stationary phase at around OD_{600}=6 (Fig. 4.2) as bands were stronger in the former. Moreover, the amount of Pdr5 exceeded by far the amount of Lr34 protein, as expected for a native yeast protein.

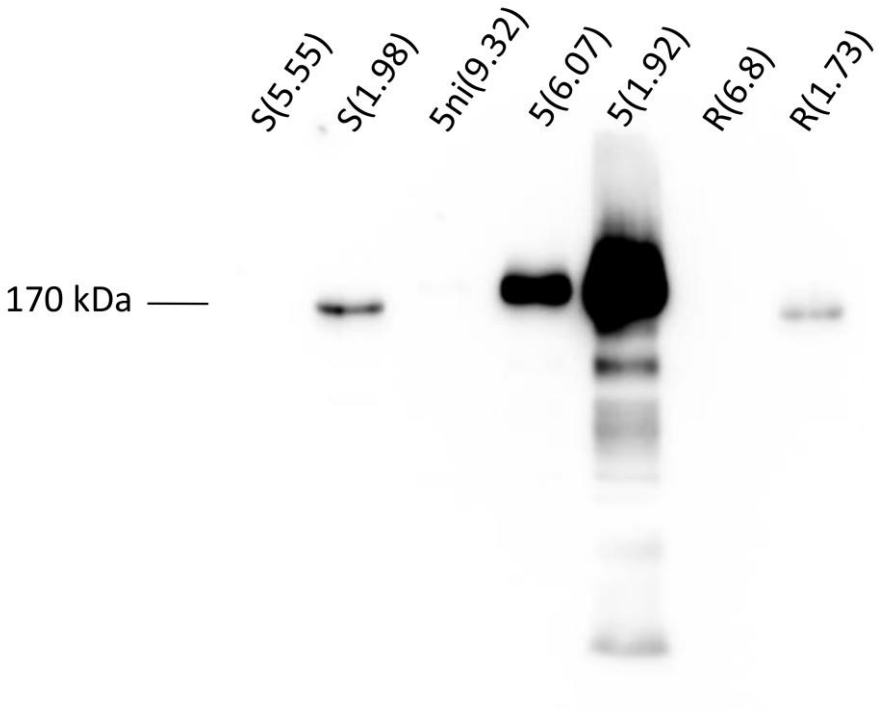

Figure 4.2 Western blot of microsomal fractions of the yeast wt strain BY4741 expressing Lr34 or *Sc*Pdr5 | Yeast cells were harvested at different culture densities (OD_{600} values in brackets) for the isolation of microsomal fractions containing Lr34sus (S), Lr34res (R) or Pdr5 (5). Non induced cells containing the Pdr5 expression vector were used as negative control (5ni).

After the determination of the optimal expression systems and conditions, protein solubilization tests were performed using different detergents as membrane proteins need to be solubilized from the membrane before affinity purifications. The anionic and non-denaturing detergents n-dodecyl β-D-maltoside (DDM) and Triton x-100 (Trit) were compared regarding their solubilization efficiency for both expression systems. The lipid composition in BY2 microsomes allowed an efficient extraction of Lr34sus protein with DDM because hardly any protein was left in the non-solubilized pellet fraction, while the supernatant containing solubilized proteins gave a strong signal (Fig. 4.3). In comparison, Triton x-100 clearly showed a lower solubilization efficiency. Unexpectedly, the sodium dodecyl sulfate (SDS)-treated sample gave a very strong signal in the pellet fraction and the detergent-free control contained a small amount of Lr34sus protein in the supernatant.

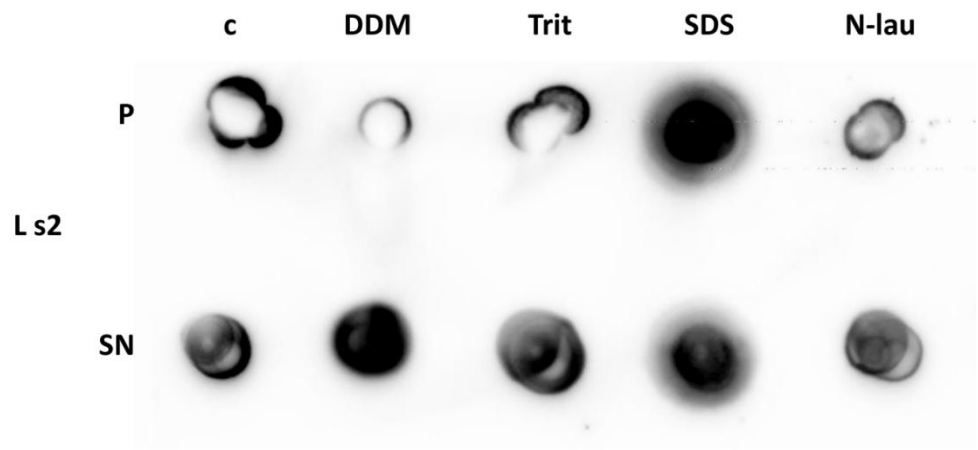

Figure 4.3 Solubilization test of BY2 microsomes containing HA-tagged Lr34sus | The microsomal fraction was isolated from BY2 cell line L s2 and tested for the solubilization of Lr34sus protein with different detergents. n-Dodecyl β-D-maltoside (DDM) and Triton x-100 (Trit) were tested as mild, non-denaturing detergents and sodium dodecyl sulfate (SDS) and N-lauroylsarcosine (N-lau) were chosen as positive control for complete solubilization. No detergent was added in the negative control (c). The upper row shows the remaining, non-solubilzed Lr34sus protein in the pellet (P), while the lower row depicts the corresponding solubilized fractions (SN).

As expected, the protein solubilization test with yeast microsomes revealed that the amount of total Lr34res protein was much lower compared to Pdr5 because only a very weak Lr34res signal could be detected in the pellet fraction of the detergent-free control (Fig. 4.4). Moreover, the solubilization of Pdr5 was very inefficient as the SN fraction of the negative control showed a stronger signal than all the detergent treated samples, which surprisingly also held true for the strong ionic detergents SDS and N-lauroylsarcosine. The only conclusion that can be drawn from this result is that Triton x-100 can be excluded as the detergent of choice for Pdr5 solubilization from yeast microsomes because of the large amount of remaining Pdr5 protein in the pellet fraction.

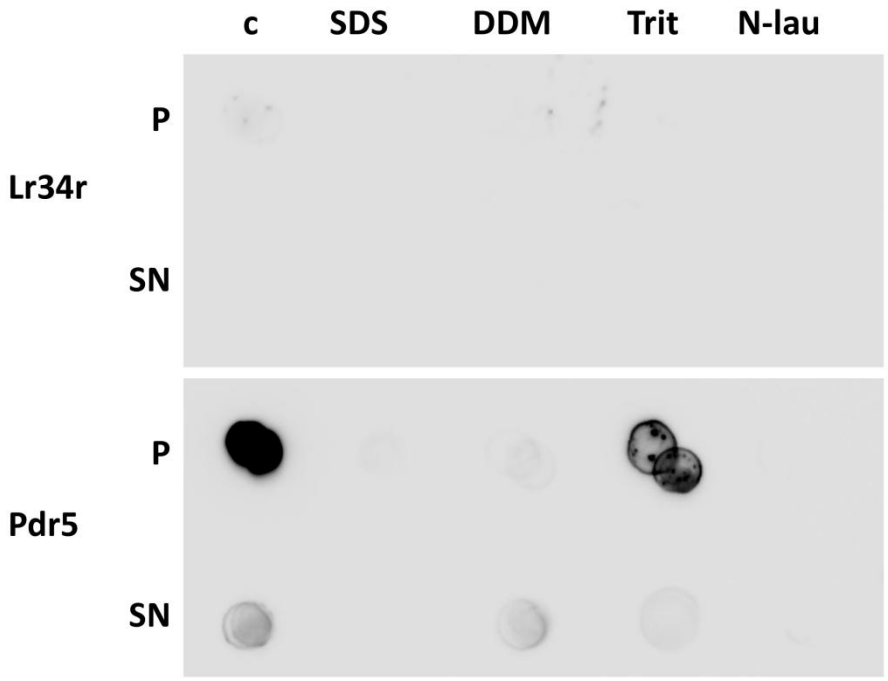

Figure 4.4 Solubilization test of yeast microsomes containing His-tagged Lr34res or Pdr5 | Microsomal fractions were either isolated from Lr34res (Lr34r) or Pdr5 expressing yeast cells and tested for protein solubilization using different detergents. n-Dodecyl β-D-maltoside (DDM) and Triton x-100 (Trit) were tested as mild, non-denaturing detergents and sodium dodecyl sulfate (SDS) and N-lauroylsarcosine (N-lau) were chosen as positive control for complete solubilization. No detergent was added in the negative control (c). The upper row shows the remaining, non solubilzed Lr34sus protein in the pellet (P), while the lower row depicts the corresponding solubilized fractions (SN).

The results of protein solubilization from BY2 and yeast microsomes revealed that the plant system should be preferred for the purification of Lr34 because of the much higher amount of obtained protein from similar culture sizes and the efficient solubilization from the membrane using DDM. Therefore, large cultures (1.8 l) of the transgenic BY2 lines L s2 and L r5 were prepared and grown to maximum density for the consecutive extraction, solubilization and affinity purification of the ABC transporter. While in the raw solubilisate (S) the HA-tagged Lr34 protein could not be detected, bands became visible after the elution from the column (E1-3) (Fig. 4.5). In addition, a small portion of the Lr34sus protein could not be solubilized according to the weak bands in the pellet fraction of L s2. However, even the amount of the eluted Lr34 protein in E2 was below the detection limit for coomassie staining (<100 ng). Only the more abundant native BY2 proteins in the other fractions were stained by the dye. The lower bands in the eluates indicate a partial degradation of the Lr34 protein.

In summary, tobacco BY2 cells are a suitable system for the expression and purification of Lr34 protein; however generation of sufficient amounts for reconstitution into liposomes will only be possible with very large cultures and an enhanced expression level of Lr34.

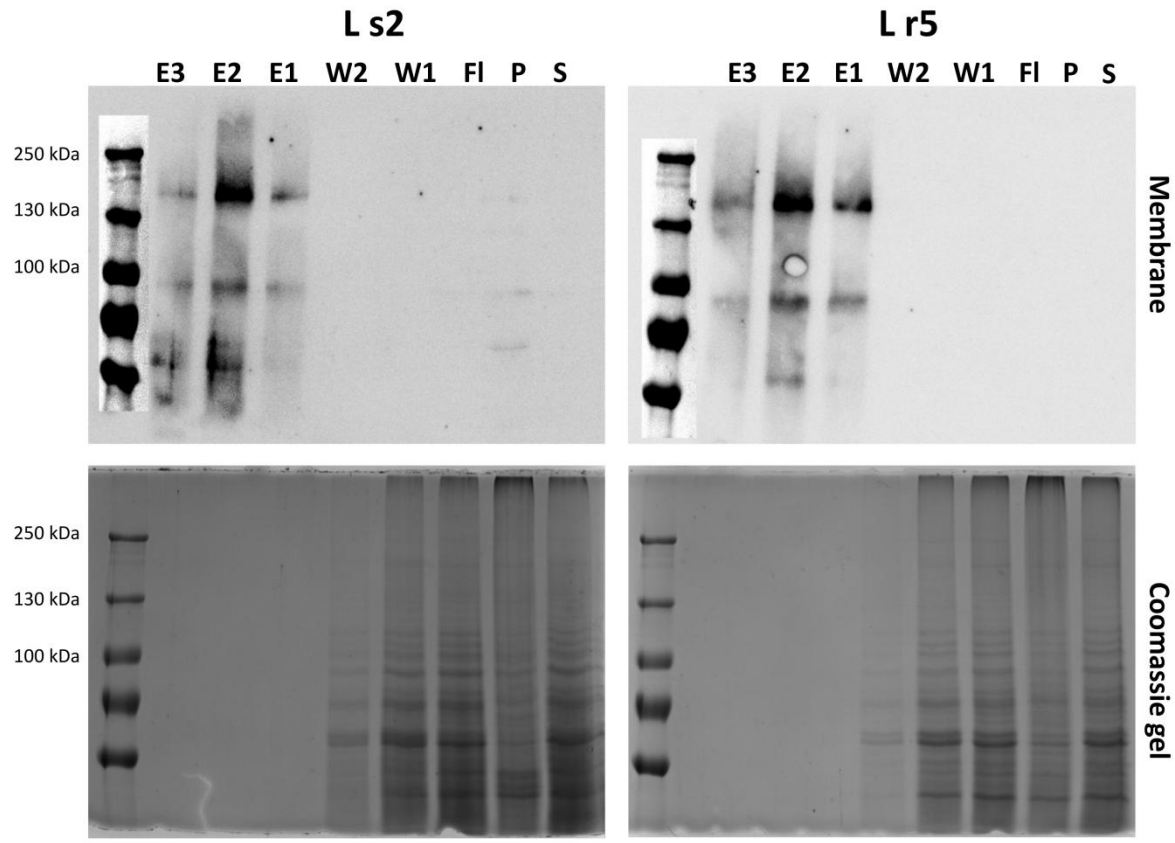

Figure 4.5 Affinity purification of HA-tagged Lr34 protein from BY2 microsomes | DDM-solubilzed microsomal fractions from large scale transgenic BY2 cell cultures (L s2 and L r5) were applied to anti-HA affinity columns for the isolation of HA-tagged Lr34 protein. At each step of the purification samples were taken for immunoblot analysis (S=solubilisate, P=pellet of non-solubilized fraction, Fl=flow through, W1=wash 1, W2=wash 2, E1=eluate 1, E2=eluate 2, E3=eluate 3). The top row depicts membranes developed with anti-HA-HRP antibodies and the lower row the corresponding coomassie-stained polyacrylamide gels.

4.3 Discussion

After the verification that the *S. cerevisae* wild type strain BY4741 and *N. tabacum* BY2 suspension cells are suitable systems for the heterologous expression of Lr34 (see chapter 2), the optimal conditions for a maximum protein yield were determined. First, large scale yeast cultures were grown to OD_{600}=2 as starting material for protein extraction. The ABC transporter Pdr5 from yeast

was expressed under the same conditions as positive control for all consecutive experiments. As expected, Pdr5 was produced to a much higher quantity than Lr34, even though both proteins have similar characteristics and sizes. Probably, these differences can be traced back to the codon usage in yeast, which is not optimal for the translation of plant proteins, considering that all ABC transporters were expressed from the same genetic constructs. Nevertheless, it was tested if the ABC transporters could be solubilized from the yeast membrane. None of the solubilisates showed a sufficiently strong signal for Pdr5 meaning either that the protein is too strongly diluted in these fractions or that the solubilization was not efficient. Presumably, SDS, N-lauroylsarcosine and also DDM are able to extract Pdr5 from the membrane as no signal could be detected in the pellet fractions and as the closely related *S. cerevisiae* ABCG transporter Pdr11 could recently be solubilized and purified successfully from yeast membranes (Laub *et al.*, 2017). Nevertheless, the absence of visible Pdr5 signals in the corresponding supernatant fractions does not allow to draw any clear conclusions. In the end, the aim was to purify Lr34 in large amounts for reconstitution into liposomes. Unfortunately, no Lr34-derived signal at all could be detected in the solubilization test, most likely as a consequence of very low expression levels and too strong dilution of the fractions. Even if a high protein yield could be achieved by the optimization of the codon usage, it would still be uncertain if Lr34 could be solubilized from yeast membranes in analogy to Pdr5. This is the principal reason why BY2 cells were chosen as preferred expression system.

The two transgenic *N. tabacum* BY2 cell lines showing the strongest signals for Lr34sus and Lr34res were selected for consecutive studies. The solubilization test demonstrated that Lr34sus could be extracted quite efficiently from BY2 membranes using DDM compared to Triton x-100. This result is in agreement with the report from Toussain *et al.* (2017) demonstrating that the closely related ABC transporter *Np*ABCG5 from *Nicotiana plumbanginifolia* can be expressed in BY2 suspension cells and solubilized using DDM for consecutive affinity purification. Unexpectedly, the SDS treated sample gave a strong signal in the pellet fraction. Most likely a big portion of the Lr34sus protein was precipitated because after the addition of SDS a flocculation was observed. On the other hand the detergent free control showed some signal in the solubilisate, possibly as a consequence of too short centrifugation times or a contamination with detergent. Nevertheless, DDM was identified as an efficient agent to solubilize Lr34 from BY2 microsomes, meaning that the ABC transporter is (at least not completely) restricted to detergent resistant lipid rafts of the PM as suggested for several human ABC transporters associated with lipid transport, such as *Hs*ABCA1, *Hs*ABCB1 or *Hs*ABCC1 (Klappe *et al.*, 2009.

As a further step, large scale BY2 cultures were prepared for the extraction and affinity purification of Lr34sus and Lr34res. For this purpose, the HA-tagged Lr34 protein versions were solubilized from the

membrane using DDM and bound to an affinity column containing immobilized anti-HA antibodies. While in the raw solubilisate the tagged protein could not be detected on the membrane after development with antibodies, bands became visible in the eluate fractions meaning that Lr34sus and Lr34res could efficiently be concentrated on the column and eluted using HA peptide. However, the amount of the purified protein was not even sufficient to become visible after coomassie staining, indicating that the total amount of purified protein was below 100 ng. Considering that a reconstitution into liposomes requires a few hundred micrograms of pure protein (Geertsma *et al*., 2008), it becomes clear that the expression has to be optimized to obtain higher protein yields. For example, the codons of the *Lr34* genomic sequence could be optimized for the expression in *N. tabacum* and a polyHis-tag could be used instead of the HA-tag in order to guarantee a more efficient affinity purification. Another necessary modification would be the preparation of very large cultures (up to 100 l) as starting material.

Alternatively, Lr34 could be produced in other organisms. For instance, the methylotrophic yeast *Pichia pastoris* or insect cells (*Spodoptera frugiperda*, e.g. Sf9) have been proven to be suitable systems for the large-scale expression of numerous ABC transporters, *i.a.* of the G-subfamily (Cai & Gros, 2003; Özvegy *et al*., 2001). The advantages of *Pichia pastoris* consist in its cost effectiveness and the ability to reach very high culture densities, while insect cells allow very complex posttranslational modifications and guarantee an easy and efficient protein extraction. For these reasons, it should be tested if Lr34 can be expressed in these organisms to high levels and if the protein can efficiently be extracted and solubilized from the membrane using suitable detergents.

In the end, the aim was to perform activity assays for a detailed analysis of the substrate specificity and translocation mechanism of Lr34sus and Lr34res (see scheme in Fig. 4.6). This requires as a first step a reconstitution of the purified and solubilized protein into liposomes consisting of PC and a small amount of one specific NBD-labelled PL. The obtained proteoliposomes are then incubated in a buffer containing Mg^{2+} and ATP for the activation of the putative PL flippase. If the corresponding NBD-PL is recognized as a substrate by the PL flippase, it will be transferred from the luminal to the extraliposomal side, which corresponds to an *in vivo* translocation from the exo- to the cytoplasmic side of the membrane. After a defined incubation time dithionite is added to the proteoliposomes in order to quench the NBD-derived fluorescence of all the externally exposed NBD-PL molecules because dithionite cannot cross the liposomal membrane. The same procedure is either performed using the same proteoliposomes but without the addition of Mg^{2+} and ATP or, alternatively, using different proteoliposomes containing no proteins with PL translocator activity (as depicted in Fig. 3.10; Rajasekharan & Gummadi, 2011). By comparing the decline of the fluorescence intensity in the different preparations the activity of the PL flippase can be determined. Because if the corresponding

NBD-PL is recognized as substrate by the PL flippase, fluorescence quenching should be stimulated in the presence of Mg^{2+} and ATP as most NBD-PLs are actively transferred to the extraliposomal side and are thus accessible for dithionite quenching.

Assuming that Lr34sus/res work as PL floppases an inverse translocation of the putative substrate NBD-PLs from the extraliposomal to the luminal side should be observed upon Mg^{2+} and ATP addition. Consequently, dithionite quenching of the NBD-fluorescence should be reduced when Lr34sus/res are active. With this assay a large set of NBD-labelled PLs can be tested as potential substrates and the differences between Lr34sus and Lr34res regarding their PL substrate specificity can be investigated.

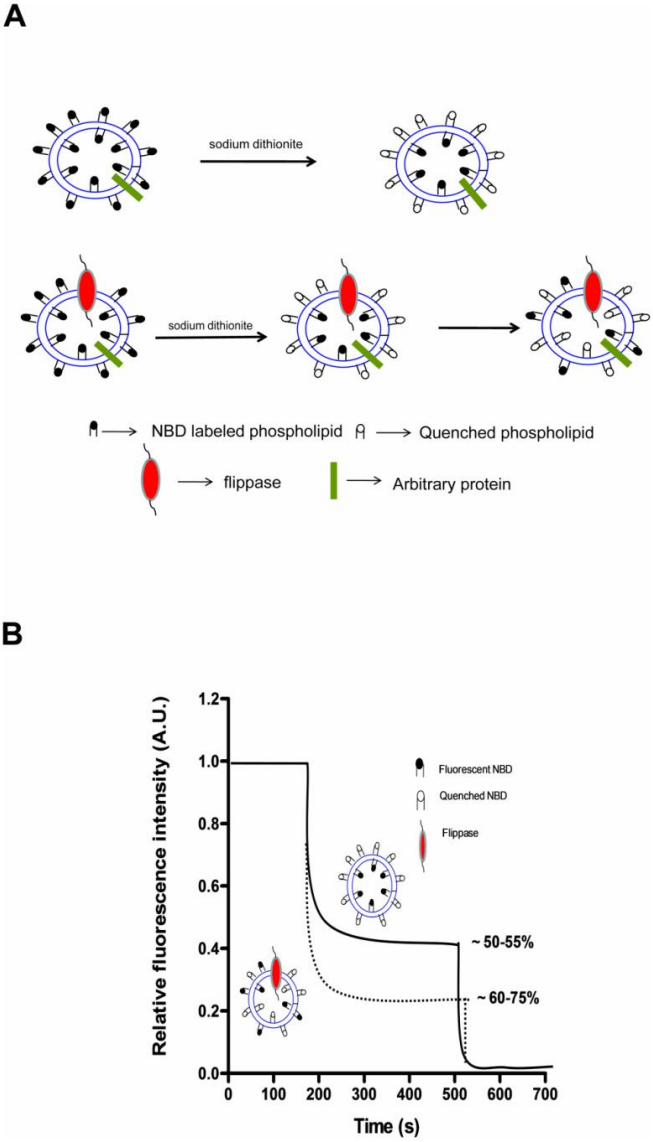

Figure 4.6 Activity assay for the determination of PL flippase activity using proteoliposomes | (a) Proteoliposomes consisting of membrane proteins with no PL translocator activity reconstituted in liposomes containing NBD-labelled PLs, which are randomly distributed within the lipid bilayer, are incubated for certain time intervalls before the fluorescence of

exposed NBD-PLs is quenched by the addition of the membrane-impermeable reducing agent sodium dithionite. The same procedure is performed for proteoliposomes containing a reconstituted PL flippase. The PL translocator activity promotes an accumulation of substrate NBD-PLs on the extraliposomal side of the lipid bilayer, which is accessible for dithionite. (b) Proteoliposomes with and without integrated PL flippases are incubated for 180 s in the presence of Mg^{2+} and ATP to stimulate PL-translocation. Afterwards sodium dithionite is added to quench the fluorescence of externally exposed NBD-PLs. As PL flippases promote the accumulation of substrate NBD-PLs on the extraliposomal side of the lipid bilayer the decline of fluorescence intensity by dithionite addition is enhanced in these proteoliposomes (source: Rajasekharan, A., & Gummadi, S. N. (2011). Flip-flop of phospholipids in proteoliposomes reconstituted from detergent extract of chloroplast membranes: Kinetics and phospholipid specificity. PloS one, 6(12), e28401)

4.4 Materials and Methods

Material

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) when not stated differently.

DNA cloning and constructs

The genomic DNA of Lr34 including N-terminal HA tag coding sequence was integrated into the p6U vector after the 35S promotor to obtain constructs for *Agrobacterium tumefaciens*-mediated transformation of BY2 cells. The p6U vector contains an *hpt* cassette for hygromycine selection in target organisms.

For the expression in yeast *Lr34* cDNA was first integrated into the $pINITIAL_{Tet}$ for a later subcloning into the destination vector pYEXNH3 containing an N-ter 10xHis tag (Geertsma & Dutzler, 2011). The same vectors were used for the cloning of *Pdr5*, which was derived from a cDNA-bank of total yeast mRNA.

All DNA constructs were verified by Sanger sequencing.

BY2 cell culture and transformation

Suspension cultures of tobacco BY2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were maintained by weekly dilution (1:10) of cells into fresh LS-medium supplemented with 30 mg/l hygromycine according to Nagata *et al.*, 1992 and cultured at 25°C with shaking at 130 rpm in the dark. The p6U vector containing *Lr34res* (genomic DNA) and N-terminal HA tag under the 35S promotor and the

empty vector, respectively, were introduced by electroporation into the *Agrobacterium tumefaciens* strain GV3101. Tobacco cells obtained from 50 ml of a 3-day-old culture were co-cultivated with 100 µl of an overnight culture of the transformed *A. tumefaciens strain* (adjusted to OD_{600}=1) on LS-plates supplemented with acetosyringone (6.6 mg/l) and hygromycin (30mg/l) for selection. After 2 days cells were collected and washed three times with BY2 culture medium and were transferred to LS-plates supplemented with cefotaxime (200 mg/l) and hygromycin (30 mg/l). After 4-6 weeks transformed BY2 cells developed calli that were checked for protein presence and used as starting material for liquid cultures.

Yeast strains and growth conditions

For the heterologous expression in *S. cerevisiae* the wt strain BY4741 (MATa his3 leu2 ura3 met15; EUROSCARF) was used. Yeast cells were transformed with the vectors for *Lr34res/sus* expression (see above) by electroporation as described in Thompson *et al.*, 1997. Liquid cultures were grown in selective SD medium (0.7% yeast nitrogen base, 2% glucose, 1x synthetic drop out media lacking uracil (Sigma-Aldrich, St. Louis, MO, USA)). For the induction of protein expression, glucose was substituted with galactose.

Yeast and BY2 microsomal membrane preparation

Fresh yeast transformants were inoculated in selective SD medium with glucose and grown overnight at 30°C with 160 rpm shaking, before inoculation into SD medium with galactose at OD_{600}=0.2. After another 6-8 h incubation period (to OD_{600}=2) under the same conditions, cells were harvested by centrifugation at 3,000 g at 4 °C for 5 min. Large cultures were then resuspended in digestion medium (1.1 M sorbitol, 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 57 u lyticase/ml) and incubated for 3 h, while small scale cultures were processed immediately by a washing step with ice-cold water. Afterwards the cell or spheroplast pellets were resuspended 1/1 (w/v) in buffer 1 (25 mM Tris-HCl pH 7.5, 5 mM EDTA, 1x protease inhibitor cocktail (PIC) (Roche, cat. No.: 11836170001)) and lysed by the addition of one volume ice-cold acid-washed glass beads (0.5 mm), followed by three cycles of 30 s homogenization with the fastprep® cell disruptor or vortex machine and 3 min rest on ice between cycles. Samples were centrifuged at 3,000 g at 4 °C for 10 min to eliminate glass beads and cell debris. After a washing step and combination of the supernatants total microsomal membrane fractions were collected by centrifugation (100,000 g; 4 °C; 45 min) and homogenized in buffer 2 (10 mM MES pH 7.8, 250 mM sucrose, 1x PIC).

Small scale BY2 cultures were harvested by centrifugation (2000 g, 8 min), while large scale cultures were separated from the culture medium by filtration through a coffee filter. After a washing step

with 1x cell vol water cells were digested in 2x cell vol enzyme solution (1% (w/v) cellulase and 0.25 % (w/v) mazerozyme in 0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$, pH 5.7) for 2.5 h. The resulting protoplasts were collected by centrifugation (100 g, 6 min), resuspended in 1x cell vol ice-cold buffer 1 (see above) and immediately homogenized with glass beads (1x cell vol) through vortexing (4 x 10 s, cooling in between). Microsomal fractions were obtained as described above and resuspended in the same buffer.

Protein quantification, coomassie staining and immunodetection

Total protein levels in microsomal fractions were determined according to Bradford, 1976 and were adjusted to each other for comparing protein expression levels.

Protein samples were run on 6.5% denaturing polyacrylamide gels for consecutive staining with coomassie brilliant blue or a protein transfer to nitrocellulose membranes. Either a rat monoclonal anti-HA-HRP coupled antibody (Roche, prod-no: 12013819001, Anti-HA-Peroxidase, High Affinity, dil: 1:1000) or a mouse monoclonal anti-polyHis-HRP coupled antibody (Sigma, prod-no: A7058, dil: 1:10,000) were used for the detection of HA-tagged and polyHis-tagged proteins, respectively. All blots were developed with luminol and enhancer solution (Advansta, WesternBright^{TM} ECL HRP substrate) and signals were detected using a Fusion-FX6 imaging device.

Solubilization tests

Total microsomal fractions were split equally before detergents were added to 1 % (w/v) final concentration (apart from negative controls). All samples were incubated for ≥30 min at 4°C under mild agitation for an efficient protein solubilization. Afterwards non-solubilized proteins were separated from the solubilisate by ultracentrifugation (100,000 g, 20 min, 4°C). The resulting pellets were resuspended in 1/10 vol buffer 2. 30 µl of each pellet and solubilisate fraction were spotted on a nitrocellulose membrane for immunodetection (see above).

Affinity purification

Solubilisates from BY2 microsomes (total protein: 24-30 mg) were loaded onto columns containing 600 µl anti-HA affinity matrix (Roche, prod-no: 11815016001) for the purification of HA-tagged Lr34 protein according to the manufacturer's instructions. Samples were taken before and at each step during the purification.

5 Reduction of transpiration and stomatal conductance in transgenic Lr34res-expressing rice plants

5.1 Introduction

The heterologous expression of *Lr34res* in related grass species not only leads to a gain of pathogen resistance but also evokes further stress responses in the corresponding plants (Chauhan *et al*., 2015; S. Krattinger, personal communication). A typical feature of *Lr34res*-expressing plants is the early development of leaf tip necrosis as a sign of premature senescence (Krattinger *et al*., 2009). Moreover, in transgenic rice transcriptome sequencing of strongly expressing lines has revealed that numerous drought-responsive genes like dehydrins or late embryogenesis abundant (LEA) proteins are upregulated in the absence of drought exposure (S. Krattinger, personal communication). According to previous results (chapter 2.3.2) showing an accumulation of phosphatidic acid (PA) in barley protoplasts, it could be assumed that Lr34res might induce the same effect in transgenic rice. Because an increase of the PA concentration at PM promotes the membrane sequestration of the protein phosphatase ABI1, an inhibitor of ABA signaling, and thus activates ABA signal transduction cascades (Zhang *et al*., 2004), which finally lead to an enhanced expression of the above-mentioned stress-responsive genes. Another hint for the involvement of PA in the induction of drought stress is the strong upregulation of PA-phosphatase observed in the transcriptomic data of *Lr34res*-expressing rice plants (S. Krattinger, personal communication) that could be interpreted as a compensatory mechanism to promote a fast turnover of the accumulating PA.

The aim of this experiment was to address whether the transcriptomic changes in fact lead to reduced transpiration rates in the corresponding rice plants. Two different transgenic lines were tested either with an intermediate expression increasing from early to late developmental stages (line 8) or a constitutively strong expression of *Lr34res* (line 19) (Krattinger *et al*., 2016). Transpiration rates, photosynthetic activity and stomatal conductance of plant can be determined using gas exchange measurements. The Licor LI-6400XT Portable Photosynthesis System uses an airtight chamber where leaves are inserted and the concentrations of the incoming and outgoing O_2, CO_2 and H_2O concentrations are measured so that all desired parameters can be calculated. This system allows highly reproducible and very sensitive gas exchange measurements on living plant tissues.

5.2 Results

The photosynthetic activity, transpiration rate and stomatal conductance of first leaves of two independent transgenic rice lines were measured with the LI-6400XT system. Interestingly, no changes were observed between young leaves (21 d) of the weakly *Lr34res*-expressing line 8 compared to the corresponding control leaves (Fig. 5.1). However, older leaves (68 d) of the same line had on average a reduced stomatal conductance, even if the difference was not significant. Transpiration rate and photosynthetic activity were always comparable between control and transgenic leaves. Finally, the strongly *Lr34res*-expressing line 19 showed a significant reduction of photosynthesis and stomatal conductance compared to the corresponding sister line. In addition, the transpiration was lower in the presence of the transgene, even though the difference was not significant.

According to the results of the gas exchange measurements a strong expression of *Lr34res* (as in line 19) induced stomatal closure causing a reduction of the photosynthetic activity and stomatal conductance. In line with this observation only older leaves of line 8, characterized by a stronger transgene expression than young leaves, showed slightly reduced stomatal conductance, even though not significant and not comparable to line 19. In summary, the *Lr34res*-promoted activation of drought stress-related gene expression in rice in fact goes along with a reduction of the stomatal conductance in the leaves resulting in decreased water loss and lower photosynthetic activity of plants showing a strong expression of *Lr34res*.

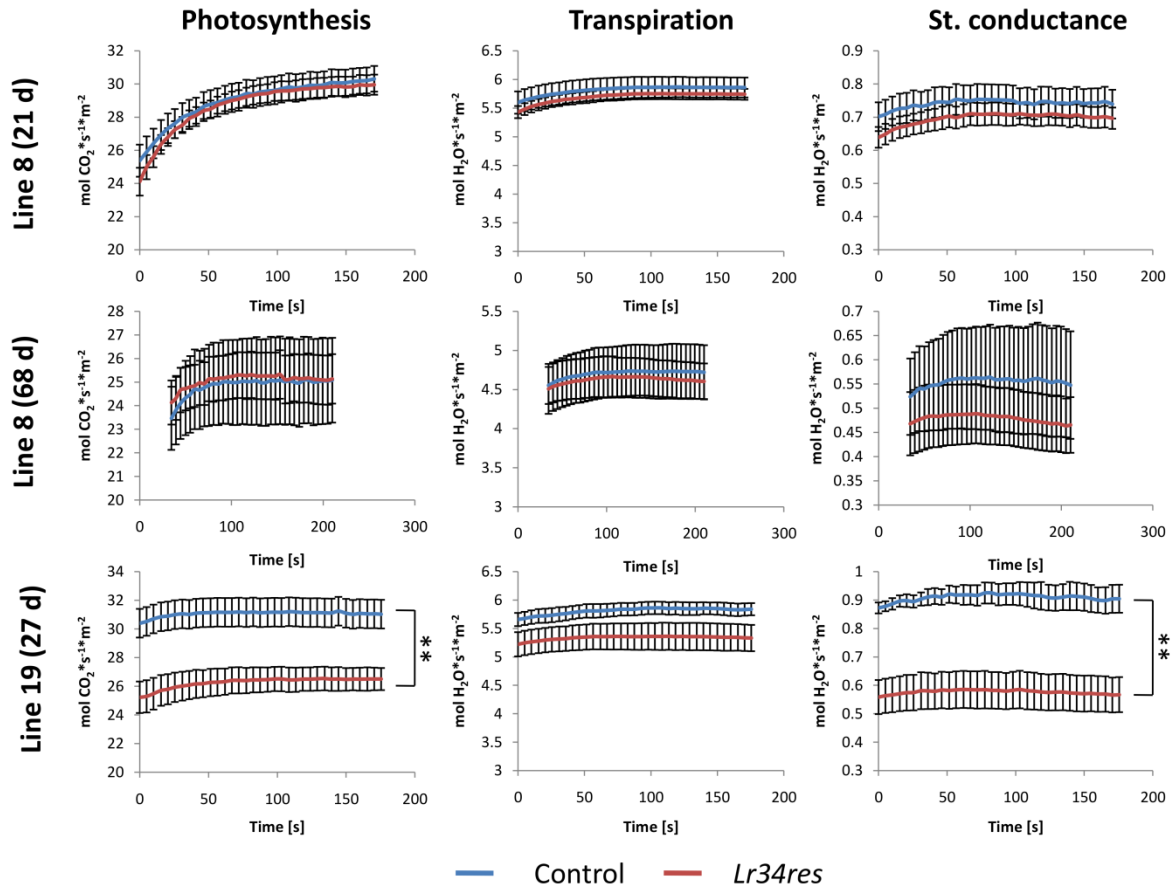

Figure 5.1 Gas exchange measurements using rice leaves | Photosynthesis, transpiration and stomatal conductance of first leaves from *Lr34res*-expressing and control plants were determined by gas exchange measurements. Line 19 was tested at 27 and line 8 at 21 and 68 days after sowing. Transgenic plants (*Lr34res*) were compared to their corresponding sister lines (control). Values are means ± s.e.m. (n=5-15), **P≤0.01, according to Student's t-test.

5.3 Discussion

It was found that the upregulation of drought-responsive genes in transgenic *Lr34res*-expressing rice lines (S. Krattinger, personal communication) correlated with reduced stomatal conductance in the leaves. The effect was most pronounced in the strongly expressing line 19, whereas only small and non-significant changes were observed for old leaves of line 8. This means that Lr34res influences stomatal regulation in a direct or indirect manner. The evidences of the previous investigations (see chapter 2) pointing to a PL flippase or floppase activity of the ABC transporter suggest that PL remodeling in the PM affects stomatal regulation. Even though a direct link has never been shown, it is known that certain PLases or, more precisely, the PL degradation products generated by these enzymes play a key role as signaling molecules in drought stress responses (Distefano *et al.*, 2008;

Wang *et al.*, 2007). The most prominent example is PLDα1, which produces PA and thereby inactivates the protein phosphatase ABI1 by its tethering to the PM (Zhang *et al.*, 2004). Consequently, the ABA signaling pathway is activated and stomatal closure is promoted. In fact, the lipid uptake experiment in barley protoplasts showed that NBD-PA is accumulating in the cytoplasmic PM leaflet in the presence of Lr34res (see chapter 2.3.2). Supposing that the same process occurs in rice cells, the activation of drought stress-responsive genes and the consecutive reduction of stomatal conductance could be explained by such an accumulation of PA. Alternatively, the ABC transporter might promote PA formation in an indirect manner either by an enhanced *de novo* biosynthesis as a consequence of its stress-inducing function similar to the situation in nitrogen-deprived algae (Wang *et al.*, 2009) or by activating certain PLases leading to increased PL turnover (Wang *et al.*, 2014). Indeed, the transcriptomic data point to alterations in lipid metabolism as many involved enzymes are strongly upregulated, *e.g.* PA-phosphatase, saccharopine dehydrogenase or glycerol-3-phosphate acyltransferase (S. Krattinger, personal communication). The enhanced levels of PA as the key intermediate of lipid biosynthesis might then affect stomatal regulation by ABI1 sequestration and the plant consecutively might counteract this effect by the upregulation of PA-phosphatase to reduce the PA levels. Alternatively, a stimulation of PLC-mediated PI(4,5)P2 hydrolysis in *Lr34res*-expressing rice might lead to Ca^{2+} release from intracellular stores and promote stomatal closure via calcium signaling (Irving *et al.*, 1992), which is supported by the biosensor studies in BY2 protoplasts pointing to a reduction of the PI(4,5)P2 content in the cytoplasmic PM leaflet in the presence of Lr34res. In conclusion, the exact mechanism behind the reduced stomatal conductance in transgenic *Lr34res*-expressing rice plants remains speculative without further studies on the content and distribution of PA and PI(4,5)P2 in guard cells. The elucidation of this mechanism would certainly help to get a better understanding of stomatal regulation in cereals and reveal the potential of genetic manipulation of PL signaling for the generation of plant species with improved drought tolerance. However, as a reduced stomatal conductance usually goes along with lower photosynthetic activity, guard cell regulation has to be balanced in a way that the negative impact on plant growth and grain yield is minimized.

5.4 Materials and Methods

Plant material

Transgenic *Oryza sativa* plants expressing *Lr34res* (line 8 and 19; Krattinger *et al*., 2016) and the corresponding sister lines were grown in a growth cabinet at 28/24°C day/night, 75% humidity and 12 h photoperiod**.**

Gas exchange measurements

Photosynthetic activity, transpiration and stomatal conductance of first leaves of transgenic rice plants were determined with the Licor machine LI-6400XT Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA) according to the manufacturer's instructions. The narrow leaf chamber was always attached to the same leaf section and experiments were always performed at the same day time in the growth cabinet to avoid diurnal fluctuations. All measurements were started shortly before constant values for the measured parameters were achieved and the following reference values were selected: StmRat: 0.5, CO2R: 500, RH_R: ≈52, PARi: 2000.

Statistical analyses

Statistical analyses for gas exchange measurements were performed using a Student's t-test with a two-tailed distribution and two-sample unequal variance

6 Conclusions and Outlook

6.1 Localization of Lr34

Even if the localization of Lr34 as a fusion construct with a fluorescent tag for microscopic protein detection could not be determined, a separation of the various subcellular membrane fractions of Lr34res-HA expressing BY2 cells on a sucrose gradient led to the discovery of an enrichment of Lr34res protein in the PM fraction. This indicates that a major part of the ABC transporter is integrated into the PM, which is consistent with previous studies confirming the PM-localization of related PDR transporter (Kang *et al*., 2010; Kobae *et al*., 2006; Crouzet *et al*., 2013) and the absence of putative signal peptides for organellar targeting in the protein sequence. However, it cannot be excluded that another part of the protein is present in other organelles, such as Golgi vesicles or endosomal compartments, indicated by the broad distribution of the Lr34res signal in the fractionation. In fact, it seems likely that – depending on the conditions – Lr34res is constantly shuttling between the trans-Golgi network and the PM as a consequence of endo- and exocytic processes, a recycling mechanism proposed for many PM-intrinsic proteins in diverse organisms, such as PIN-FORMED proteins (PINs), human beta 2-adrenergic receptors or transferrin receptors in rats (Boutté *et al*., 2006; Von Zastrow & Kobilka, 1992; Harding *et al*., 1983). Moreover, the PL translocator activity of Lr34res *per se* could have an impact on the subcellular destination because PL distribution in Golgi vesicles and the PM is a crucial factor for membrane fusion events along the secretory pathway. As a consequence, the delivery of newly synthesized Lr34 protein to the PM might be disturbed in a way that Lr34res-containing Golgi vesicles would accumulate as endosomes in the cytosol and would finally be converted to lysosomes, where the protein would be degraded. Nevertheless, the result of the membrane fractionation proved the presence of full-size Lr34res protein in the plant PM required for consecutive studies on the activity of the transporter. Although the subcellular localization of Lr34res in wheat as the original species could not be investigated, it seems likely that the protein can also be found in the PM of wheat. Moreover, the presence of Lr34sus in the PM has not yet been proven. Presumably, the two mutations in the first TMD do not affect the targeting of the ABC transporter even though a different PL translocator activity compared to Lr34res might have an impact on its turnover-rate and thereby the steady-state level of Lr34sus in the PM as discussed above.

6.2 Potential impact of Lr34sus- and Lr34res-mediated PL redistribution in the PM on cellular signal transduction

Based on previous observations, such as neutral lipid accumulation in transgenic barley leaves and the *S. cerevisiae Sec14*^{ts}-mutant or the upregulation of genes involved in lipid metabolism in transgenic barley (Chauhan *et al*., 2015) upon the expression of *Lr34res*, it was assumed that the ABC transporter affects lipid metabolism. From the literature it is known that several ABC transporters of various subfamilies work as translocators for certain lipids from one side of the membrane to the other, a process called lipid flipping (Decottignies *et al*., 1998; Bodzioch *et al*., 1999; Krishnamurthy *et al*., 2002; Woehlecke *et al*., 2003; Molday *et al*., 2009; Tarling *et al*., 2013; Wiesinger *et al*., 2013). In most cases the lipids are transferred from the cytoplasmic to the exoplasmic membrane leaflet. Therefore, functional assays were performed in different heterologous systems with the aim to investigate if the ABC transporter versions Lr34sus and Lr34res also possess such an activity. Indeed, Lr34sus and Lr34res either directly or indirectly changed the distribution of several membrane lipids. Especially PS was found to be exposed in the PM meaning that the transporters work as PS floppases. Moreover, PA accumulated in the cytoplasmic PM leaflet in *Lr34res*-expressing barley protoplasts, which can either be interpreted as a direct Lr34res-mediated translocation of PA or a compensatory mechanism for the loss of PS on the inner side of the PM. In this case PA would not only compensate for the loss in space but also for the local loss of negative charge in the PM, which is of outstanding importance for the associated proteome and the function of PM-intrinsic proteins. In addition to the approaches with fluorescence-labeled PLs, biosensors for the specific detection of various native PLs were expressed in transgenic *Lr34res*-expressing tobacco BY2 protoplasts in order to localize the corresponding membrane lipids and detect putative changes in their distribution caused by the activity of the transporter. Beside the confirmation of a PS reduction, this method allowed in addition the detection of significantly reduced PI(4,5)P2 levels in the cytoplasmic PM leaflet of *Lr34res*-expressing protoplasts. However, without the external application of purified PI(4,5)P2-biosensor it cannot be determined if this PL is translocated to the exoplasmic PM leaflet by Lr34res or if these changes are caused by alterations in phosphoinositide metabolism either through enhanced enzymatic conversion by phospholipase C (PLC) or through reduced biosynthesis by PI(4)P-kinase, since the activity of these two enzymes depends on the cytoplasmic PS and PA concentrations, respectively, of the PM (Moritz *et al*., 1992; Ananthanarayanan *et al*., 2002). For instance, a Lr34res-mediated reduction of the cytoplasmic PS level might force the cell to produce more PLC molecules for compensation, leading to enhanced PI(4,5)P2 turnover. Considering that many PLases are up- or downregulated under biotic and abiotic stress conditions (Munnik *et al*., 1998; Wang *et al*., 2002;

Yamaguchi *et al*., 2009; Zhao *et al*., 2013; Zhao, 2015), a detailed analysis of their activity depending on the presence and absence of Lr34res would be informative to resolve the involved physiological pathways which lead to the observed phenotypes in transgenic plants. Finally, Lr34sus and Lr34res were expressed in yeast to check for putative changes in PL distribution in the yeast PM caused by the activity of both transporter versions. Even if the yeast membrane lipid composition is different from plants, the exposure of PS could also be detected in this system by an increase of the sensitivity to the PS-specific toxin papuamide A in *Lr34sus*- and *Lr34res*-expressing yeast cells. Interestingly, the opposite effect was observed for duramycin, which specifically binds to exposed PE. This means that at least in yeast Lr34sus and especially Lr34res promote the translocation of PE from the outer to the inner side of the PM. The different lipid composition or the expression level of the ABC transporter might explain why PE translocation was observed in yeast and not in barley protoplasts. The upregulation of PE-binding proteins in *Lr34res*-expressing wheat (Hulbert *et al*., 2007) and transgenic rice (S. Krattinger, personal communication) leaves is a good hint that PE might indeed be a substrate of the transporter. In the end, a complete and explicit analysis of the substrate spectra of Lr34sus and Lr34res as well as a functional distinction between the two transporter versions can only be performed using proteoliposomes containing pure protein and a defined lipid composition, as discussed before. However, it is obvious that the changes of PL distribution within the PM – especially the removal of PS from the cytoplasmic leaflet – have severe consequences for the membrane-bound proteome inside and outside of the cell.

PS exposure is a universal mechanism associated with apoptosis in many cell types (Woelecke *et al*., 2003; O'Brien *et al*., 1997), which might explain the early emergence of senescence in *Lr34res*-expressing plants. As this process is always associated with a reduction of intracellular carbohydrate levels, the growth of invading biotrophic pathogens might be severely impaired by the scarce availability of carbohydrates as nutrition source. Moreover, the PS floppase activity of Lr34res might counteract the uptake of fungal effector proteins that potentially use the inward translocation of PS for host cell entry, a mechanism that has already been proposed for PIP-binding effector proteins (Kale *et al*., 2010), even though controversially discussed (Gan *et al*., 2010; Yaeno *et al*., 2011). A reduction of the freely available carbohydrates and the inhibition of fungal effector entry are possible explanations for the strongly delayed fungal spread in leaf tissue of *Lr34res*-expressing plants because biotrophic fungi rely on their secreted effector proteins for the successful establishment of haustoria for the uptake of nutrients from the host plant. In addition, biotrophic pathogens cannot evolve any mechanism, such as effector-triggered host susceptibility, to overcome these hurdles, which would also explain the durability of *Lr34res*-mediated resistance in cereals.

Inside the cell the activity of Lr34 would promote the detachment of PS-binding proteins with signaling function from the PM and consequently modulate their activity (Fig. 6.1, Table 2, Stace & Ktistakis, 2006). For example, many PLC classes have a C2 domain for the specific recognition of PS via electrostatic interactions with the negatively charged PS (Ananthanarayanan *et al*., 2002). The recruitment of PLC to the PM stimulates its activity and consequently the hydrolysis of PI(4,5)P2 to DAG and IP_3 that act as signaling molecules and evoke various cellular responses, such as the release of Ca^{2+} from internal stores. This mechanism might be one reason for the observed reduction of stomatal conductance in leaves of *Lr34res*-expressing rice plants assuming a burst of the cytosolic Ca^{2+}-concentration in guard cells. Not only PLC but also the evolutionarily highly conserved copines are capable of binding PS, which likely alters their activity (Creutz *et al*., 1998; Tomsig & Creutz, 2000). Even though their precise molecular function has not been resolved so far, they are known to play critical roles in development and disease resistance (Jambunathan *et al*., 2001; Zou *et al*., 2016). In addition, recent reports have demonstrated that copines are important regulators of ABA- and pathogen-triggered stomatal closure (Yang *et al*., 2017), suggesting that Lr34res-induced alterations in copine activity might at least partially be responsible for the increased pathogen resistance (Krattinger *et al*., 2016) and the observed reduced stomatal conductance in transgenic *Lr34res*-expressing rice plants. Finally, the activity of copines affects programmed cell death and defense responses in *Arabidopsis thaliana* (Jambunathan *et al*., 2001). A missregulation of copine-activity caused by PS translocation in the PM would thus be a possible explanation for the premature senescence in *Lr34res*-expressing plants. Another important PS-binding protein is the diacylglycerol kinase, which is a prominent regulatory factor in lipid metabolism. Considering that Lr34res is removing PS from the cytoplasmic membrane leaflet, the activity of DAG kinase might be modulated in a way that the PA/DAG equilibrium is shifted. As a compensatory mechanism the putative PA deficit could be balanced by an induction of PL degrading enzymes, *e.g.* PLD, forming PA from membrane lipids. The resulting elevation of the PA level in such a way would explain the activation of the typical PA-associated responses in *Lr34res*-expressing plants. Finally, sphingosine kinase is another important factor involved in cellular signaling, apoptosis and senescence and was shown to interact with PS despite a lack of known PS-binding domains (Olivera *et al*., 1996). The phosphorylation of certain sphingolipids, especially sphingosine, phytosphingosine and ceramide, is *i.a.* crucial for the regulation of ABA-responses for stomatal closure or the facilitation of cold tolerance (Ng *et al*., 2001; Chen *et al*., 2012). Moreover, sphingolipids fulfill critical functions in defense against bacterial and fungal pathogens by inducing programmed cell death, which can be observed even in the absence of pathogens upon the intracellular accumulation of ceramide (Liang *et al*., 2003). Under these considerations it could be assumed that membrane remodeling by Lr34res

not only affects PLs but might also alter sphingolipid levels or – more precisely – their phosphorylation state leading to the observed activation of abiotic and biotic stress responses.

On the other hand the Lr34res-mediated increasing PA concentration in the cytoplasmic PM leaflet presumably attracts proteins that have the ability to bind PA and thereby modulate their activity (Fig. 6.1, Table 1, Stace & Ktistakis, 2006). The most prominent example in plants is the protein phosphatase ABI1 (ABA insensitive 1), a crucial component of the ABA signaling pathway. The perception of ABA leads to the activation of a specific PLD that converts structural membrane lipids into PA. This local PA accumulation is sensed by ABI1, an inhibitor of the ABA signaling pathway, whereupon it gets attracted to the PM and thereby loses its inhibitory function resulting in stomatal closure (Zhang *et al*., 2004). It seems likely that this mechanism causes the reduction of stomatal conductance in transgenic *Lr34res*-expressing rice plants. Another important PA-binding protein is the phosphoinositide dependent kinase 1 from *A. thaliana* (*At*PDK1). At least in animals PDK1 is known to integrate signals coming from different receptors and interact for example with several cAMP-dependent or cGMP-dependent protein kinases finally leading to adaptations in growth, cell division or apoptosis (Storz & Toker, 2002). As these signaling pathways are highly conserved among almost all organisms, PDK1 probably has similar functions and interaction partners in plants. The alteration of PDK1 activity by an Lr34res-mediated increase of the cytoplasmic PA concentration in the PM might for example explain the early emergence of senescence in *Lr34res*-expressing plants.

Table 1
Phosphatidic acid binding proteins

Protein	Function	Location	PA binding region	Notes	References
Plants					
ABI1	Protein phosphatase	Cytosol	ESRKVLISRINSPNLMKESAAADIVVVDISAG	PA inhibits activity	[24]
AtPDK1	Protein kinase	Cytosol	PH domain at residues 391–491	PA enhances activity	[82]
PEPC	Phosphoenolpyruvate carboxylase	Cytosol	Unknown	Unknown	[83]

Aminoacids which have been shown to be directly involved in PA recognition have been underlined.

Table 2
Phosphatidylserine binding proteins

Protein	Function	Crystal structure with PS-binding information[a]	PS binding domains	Requires Ca?	Predominant PS interactions	Other references
Annexins	Unclear. Implicated in membrane trafficking, inhibition of coagulation, and signal transduction.	Yes [63,64]	Annexin Core	Yes	Non-specific electrostatic and membrane penetration by hydrophobic residues. Serine headgroup specific interactions	[62,65,84]
PLC	Hydrolyses PI-4,5-P_2 to IP_3 and DAG, in receptor-coupled signaling from plasma membrane	Yes [51,88]	C2 domain (PKC-like)	Yes	Evidence for serine-headgroup specific electrostatic interactions, and possible membrane penetration by hydrophobic residues	[52,89,90]
Copines	Unknown	No	2× C2 domains (PKC-like)	Yes	Unknown	[94,95]
NO synthase	Generates NO (an intracellular messenger)	No	None	No	Non-specific electrostatic and hydrophobic. Formation of amphipathic helix	[114,115]
DGK	Phosphorylates DAG to form PA	No	None	?	Non-specific electrostatic. Possible formation of amphipathic helix	[116,117]
SK1	Phosphorylates sphingosine	No	None	No	Electrostatic?	[20,22]

[a] Structure with PS, PS analogue, or without PS but with detail of PS binding site.

Figure 6.1 Examples of PA and PS-binding proteins present in plants | Several important cytosolic proteins have PA-and PS-binding mudules that that allow their binding to the corresponding PL under certain conditions, *e.g.* high Ca^{2+}-concentrations. They can be found in almost all organisms and examples from plants with proven PL-binding capacity are depicted in these two tables. Abbreviations: ABI1, ABA insensitive 1; PDK1, phosphoinositide dependent protein kinase 1; PLC, phospholipase C; PKC, protein kinase C; DGK, diacylglycerol kinase; SK1, sphingosine kinase 1. (adapted from: Stace, C. L., & Ktistakis, N. T. (2006). Phosphatidic acid-and phosphatidylserine-binding proteins. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1761(8), 913-926.)

The postulated mechanism of PL translocation by Lr34 and the consequences for the membrane-associated proteome are summarized in the model in Fig. 6.2. Briefly, changes in PL distribution evokes the activation/deactivation of specific signal transduction cascades via specific PL-binding proteins finally leading to the expression of stress-responsive genes. Exposed PS can be recognized by external proteins either coming from the plant itself or from potential invading pathogens.

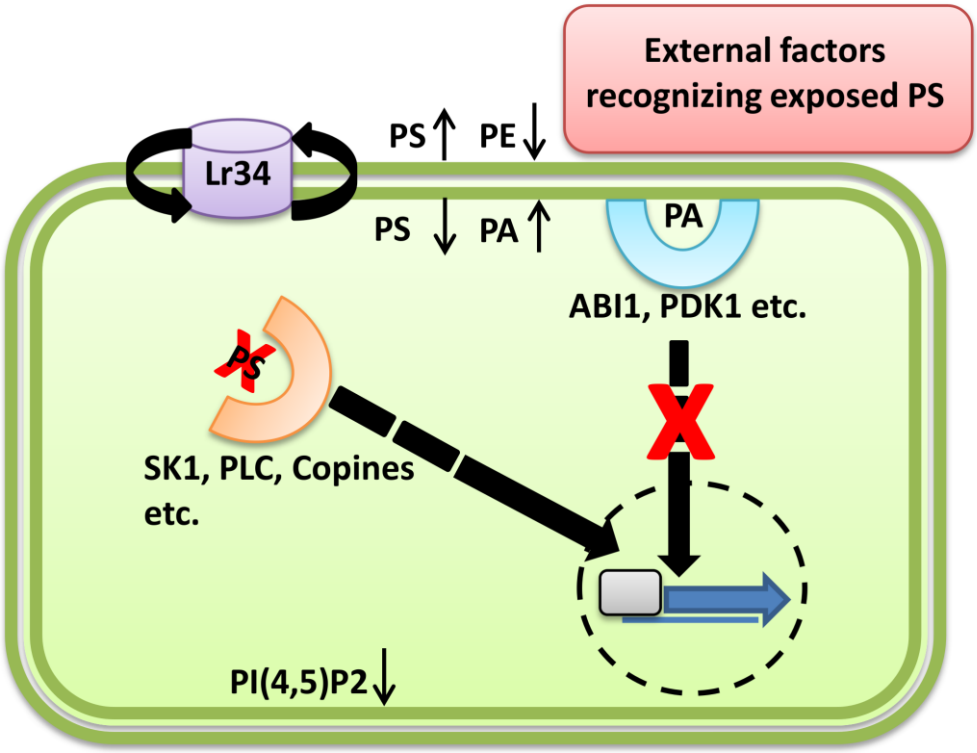

Figure 6.2 Model of proposed Lr34 mechanism | In the PM Lr34 works as translocator for certain PLs leading to enhanced PS exposure, while the levels of cytoplasmic PS and PI(4,5)P2 are reduced. In contrast, the transfer of PA (and PE) from the exo- to the cytoplasmic membrane leaflet seems to be increased. Consequently, PS- or PI(4,5)P2-binding proteins are detached from the PM and PA-binding proteins are attracted to the PM leading to the activation or deactivation of cellular signal transduction cascades with impact on the expression of stress-responsive genes. In addition, external factors either derived from the plant itself or potentially also from invading pathogens might recognise the exposed PS, which could be a crucial factor for *Lr34res*-mediated pathogen resistance. Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; ABI1, ABA insensitive 1; PDK1, phosphoinositide dependent kinase 1; SK1, sphingosine kinase 1; PLC, phospholipase C; PI(4,5)P2, phosphatidylinositol-(4,5)-bisphosphate.

6.3 Consequences of Lr34 activity for cellular lipid metabolism

The interbilayer translocation of lipids is an important process not only for the maintenance of PL asymmetry in the PM but also as an inducer of vesicle formation in the secretory pathway. More precisely, PL flippases transfer PE and PS from the exoplasmic to the cytoplasmic membrane leaflet at the sites of emerging vesicles in the Golgi network, which creates membrane curvature stress and in addition promotes the binding of coat proteins to allow efficient vesicle budding (Graham, 2004). Supposed that Lr34sus/res counteract this process by a reverse translocation of the corresponding PLs, it can be assumed that vesicle formation is hampered with negative impact on the whole

secretory pathway. This would for example be another possible explanation for the growth retardation in *Lr34sus/res*-expressing yeast cells apart from a missregulation of the cell cycle.

In addition, massive accumulation of neutral lipids (TAG) was observed in transgenic barley plants as well as in the *Sec14*^{ts} PL transfer protein yeast mutant through *Lr34res*-expression. Often lipid accumulation in oil bodies is a cellular reaction to adverse environmental conditions or pathogen attack (Solovchenko, 2012). Presumably, Lr34 somehow mimics stress exposure and thereby promotes the formation of oil bodies. Interestingly, in contrast to wild type yeasts the *Sec14*^{ts} mutant already accumulates lipids under standard growth conditions (unpublished observation), which indicates that oil body formation can obviously be linked to defects in PL transfer in the secretory pathway. If the concentration of certain toxic lipid intermediates, *e.g.* free FAs, as a consequence of disturbed inter-membrane transfer exceeds a certain limit, they could be sequestered in oil bodies for deposition and further metabolization, respectively. Free FAs can for example be derived from membrane lipid degradation by PLA. This mechanism might explain the accumulation of neutral lipids in *Lr34res*-expressing barley plants (Bucher, 2017) and in the PL transfer-deficient yeast mutant (chapter 2.3.4).

In order to check for such an enhanced turnover of PLs in the presence of Lr34res, barley protoplasts were incubated with several different NBD-labelled PLs to observe their PLase-dependent degradation. Unfortunately, only a small number of PLs could be tested in this assay, but as most PLases have broad substrate spectra (even though they usually have a preference for PLs with a specific head group), it was assumed that at least the major membrane lipids PC and PE are recognized by some barley PLases. In addition, the NBD-labelled PA and PS derivatives were tested for an enhanced Lr34res-dependent turnover. The final result showed that PA, PC and PE were converted to PA and DAG, respectively, at similar rates in control and *Lr34res*-expressing barley protoplasts meaning that the activity of PLC and PLD species recognizing these PLs as substrates is not altered in the presence of the ABC transporter. Nevertheless, it must be considered that the levels of degraded PLs were quantified after 16 h incubation for the determination of long-term effects. Previous studies demonstrated, however, that PA is metabolized very quickly in barley protoplasts (unpublished observations), resulting in strong DAG-accumulation, probably to prevent the cellular activation of PA-signaling pathways by excessive PA. Yet, no indications were found that the activity of Lr34res has any influence on this rapid accumulation of DAG in spite of the enhanced PA level in *Lr34res*-expressing barley protoplasts after 10 min incubation. The only differentially metabolized PL was PS, indicated by a lower conversion rate of PS to PA in the presence of the ABC transporter. Considering that Lr34res is translocating PS from the cytoplasmic to the exoplasmic PM leaflet and thereby putatively shields it from degradation by PLases, this result was however not

surprising. Consequently, it cannot be concluded that PLase activity is altered in *Lr34res*-expressing barley, although other membrane lipids, such as PIPs, can very well be affected in their turnover. Especially phosphoinositide-specific PLC activity depends on the presence of PS in the cytoplasmic leaflet of the PM (Lomasney *et al*., 1999), so that an Lr34res-mediated decrease of the cytoplasmic PS level would have a severe impact on the activity of this enzyme and the PI(4,5)P2 concentration in the PM. Unfortunately, NBD-labelled PIPs are not commercially available and could thus not be included in PLase activity assay. An increased turnover of these PL-species in the presence of the ABC transporter would certainly explain the activation of cellular signal transduction cascades upon DAG and IP_3 generation but the strong accumulation of neutral lipids in barley and the yeast mutant presumably cannot be related to this catabolic process because of the low abundance of PIPs. Instead, the source of these neutral lipids chould rather be found in an enhanced *de novo* biosynthesis of TAG as a consequence of stress induction by Lr34res. This hypothesis is supported by the upregulation of several enzymes involved in lipid metabolism in transgenic *Lr34res*-expressing rice and barley plants, *e.g.* saccharopine dehydrogenase, sec14-like lipid-transfer proteins, phosphatidic acid phosphatase, etc. (S. Krattinger, personal communication; Chauhan *et al*., 2015).

6.4 Manipulation of PL metabolism for improved biotic and abiotic stress tolerance in plants

In the last years it has become clear that PLs and PLases are crucial factors in mediating cellular adaptations to changing environmental conditions. The mechanism of PLase activation upon physical or hormone stimulation is highly conserved among organisms and always involves the generation of lipid-derived signaling molecules, such as PA, DAG or IP_3. Numerous reports have demonstrated that the overexpression of certain PLases evokes various physiological responses, which are associated for example with enhanced drought tolerance or pathogen resistance (Munnik *et al*., 1998; Wang *et al*., 2002; Yamaguchi *et al*., 2009; Zhao *et al*., 2013; Zhao, 2015). Especially in plants these enzymes are of outstanding importance, which is reflected in the high number of PLD isoforms (17 in *Oryza sativa*) compared to animals (2 in *Homo sapiens*) or yeast (1 in *Saccharomyces cerevisiae*). However, considering that lipid modifications at the PM upon receptor stimulation by external factors are upstream of many diverse intracellular signal transduction cascades it becomes clear that alterations in PLase activity usually evoke very complex physiological responses leading to phenotypes with favorable and unfavorable traits. Consequently, instead of overexpressing or silencing PLases the cellular targets of genetic manipulation should be chosen further downstream, *e.g.* by modulating

the activity/expression level of PL-binding proteins that are involved in only one specific signal transduction cascade. For example, an enhancement of the PA-affinity of the inhibitory protein phosphatase ABI1 would be expected to constantly induce ABA-signaling with a potentially positive effect on drought tolerance. Such an approach would be more targeted than the elevation of the intracellular PA level (*e.g.* by overexpressing PLDs), which would cause additional physiological responses. The same holds true for alterations in the subcellular distribution of certain PLs, which is the supposed mechanism of Lr34res in promoting pathogen resistance and drought tolerance. Because the translocation of PS from the cytoplasmic to the exoplasmic PM leaflet presumably causes the activation of several distinct stress responses as a consequence of changes in the PM-associated proteome (see above). Even if this mechanism is an efficient way to provide broad-spectrum and durable resistance against biotrophic pathogens, the negative impact on the overall plant performance (early senescence, high energy demand for TAG biosynthesis, reduced grain yield etc.) can be quite dramatic, especially when the expression level of *Lr34res* is very high, as observed in transgenic barley plants (Risk *et al*., 2013). In contrast, the negative impact of Lr34res activity seems to be physiologically balanced when heterologously expressed in durum wheat (*Triticum turgidum*), as no symptoms of premature senescence were observed even in strongly expressing lines (Rinaldo *et al*., 2017). Consequently, the only way to use the ABC transporter for crop engineering is the generation of plants with controlled levels of *Lr34res*-expression in a background allowing a physiological compensation of potential negative side-effects while providing efficient pathogen resistance. Moreover, it would be highly informative to resolve the structural and functional differences between Lr34res and Lr34sus because this would certainly help to understand the precise mechanism behind the pathogen resistance. For example, the two mutations in the first transmembrane domain of the ABC transporter might cause a shift in the substrate specificity or -preference for certain PLs, which are crucial for host-pathogen interactions. This knowledge could then be used for manipulating the function of related ABC transporters from other crop species in order to evoke pathogen resistance in a similar way.

7 Literature

Abuznait, A. H., & Kaddoumi, A. (2012). Role of ABC transporters in the pathogenesis of Alzheimer's disease. *ACS chemical neuroscience, 3*(11), 820-831.

Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., & Holthuis, J. C. (2006). Loss of P4 ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. *Molecular biology of the cell*, *17*(4), 1632-1642.

Ananthanarayanan, B., Das, S., Rhee, S. G., Murray, D., & Cho, W. (2002). Membrane targeting of C2 domains of phospholipase C-δ isoforms. *Journal of Biological Chemistry, 277*(5), 3568-3575.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant physiology*, *24*(1), 1.

Athenstaedt, K., & Daum, G. (1999). Phosphatidic acid, a key intermediate in lipid metabolism. *The FEBS Journal, 266*(1), 1-16.

Balboa, M. A., Balsinde, J., Dillon, D. A., Carman, G. M., & Dennis, E. A. (1999). Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *Journal of Biological Chemistry*, *274*(1), 522-526.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology, 37*(8), 911-917.

Bodzioch, M., Orsó, E., Klucken, J., Langmann, T., Böttcher, A., Diederich, W., ... & Kaminski, W. E. (1999). The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nature genetics, 22*(4), 347.

Borst, P., Zelcer, N., & Van Helvoort, A. (2000). ABC transporters in lipid transport. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1486*(1), 128-144.

Borths, E. L., Poolman, B., Hvorup, R. N., Locher, K. P., & Rees, D. C. (2005). In vitro functional characterization of BtuCD-F, the *Escherichia coli* ABC transporter for vitamin B12 uptake. *Biochemistry*, *44*(49), 16301-16309.

Boutté, Y., Crosnier, M. T., Carraro, N., Traas, J., & Satiat-Jeunemaitre, B. (2006). The plasma membrane recycling pathway and cell polarity in plants: studies on PIN proteins. *J Cell Sci, 119*(7), 1255-1265.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.

Bradshaw, R. A., & Dennis, E. A. (Eds.). (2009). *Handbook of cell signaling*. Academic press.

Braun, V., & Hantke, K. (2011). Recent insights into iron import by bacteria. *Current opinion in chemical biology*, *15*(2), 328-334.

Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Ødum, N., ... & Mundy, J. (2002). Knockout of *Arabidopsis* accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes & Development*, *16*(4), 490-502.

Bucher, R. R. (2017). Mass-spectrometry based metabolomic approaches to characterize plant pathogen resistance in cereals and the flavonols modifying development in *Arabidopsis thaliana*. Universität Zürich, [010899177].

Cai, J., & Gros, P. (2003). Overexpression, purification, and functional characterization of ATP-binding cassette transporters in the yeast, *Pichia pastoris*. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1610*(1), 63-76.

Calon, F., & Cole, G. (2007). Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *77*(5-6), 287-293.

Chauhan, H., Boni, R., Bucher, R., Kuhn, B., Buchmann, G., Sucher, J., ... & Glauser, G. (2015). The wheat resistance gene Lr34 results in the constitutive induction of multiple defense pathways in transgenic barley. *The Plant Journal, 84*(1), 202-215.

Chen, J., Sharma, S., Quiocho, F. A., & Davidson, A. L. (2001). Trapping the transition state of an ATP-binding cassette transporter: evidence for a concerted mechanism of maltose transport. *Proceedings of the National Academy of Sciences*, *98*(4), 1525-1530.

Chen, M., Markham, J. E., & Cahoon, E. B. (2012). Sphingolipid Δ8 unsaturation is important for glucosylceramide biosynthesis and low-temperature performance in *Arabidopsis*. *The Plant Journal*, *69*(5), 769-781.

Coleman, J. A., Kwok, M. C., & Molday, R. S. (2009). Localization, purification, and functional reconstitution of the P4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. *Journal of Biological Chemistry*, *284*(47), 32670-32679.

Conseil, G., Decottignies, A., Jault, J. M., Comte, G., Barron, D., Goffeau, A., & Di Pietro, A. (2000). Prenyl-flavonoids as potent inhibitors of the Pdr5p multidrug ABC transporter from *Saccharomyces cerevisiae*. *Biochemistry*, *39*(23), 6910-6917.

Coursol, S., Fan, L. M., Le Stunff, H., Spiegel, S., Gilroy, S., & Assmann, S. M. (2003). Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins. *Nature*, *423*(6940), 651.

Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J., & Cohen, J. (1998). The copines, a novel class of C2 domain-containing, calciumdependent, phospholipid-binding proteins conserved from *Paramecium* to humans. *Journal of Biological Chemistry*, *273*(3), 1393-1402.

Crouzet, J., Roland, J., Peeters, E., Trombik, T., Ducos, E., Nader, J., & Boutry, M. (2013). NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. *Plant molecular biology, 82*(1-2), 181-192.

Curwin, A. J., Fairn, G. D., & McMaster, C. R. (2009). Phospholipid transfer protein Sec14 is required for trafficking from endosomes and regulates distinct trans-Golgi export pathways. *Journal of Biological Chemistry*, *284*(11), 7364-7375.

Dave, A., & Graham, I. A. (2012). Oxylipin signaling: a distinct role for the jasmonic acid precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA). *Frontiers in plant science*, *3*, 42.

Decottignies, A., Grant, A. M., Nichols, J. W., de Wet, H., McIntosh, D. B., & Goffeau, A. (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. *Journal of Biological Chemistry, 273*(20), 12612-12622.

Dennis, E. A. (2015). Introduction to thematic review series: phospholipases: central role in lipid signaling and disease. *Journal of lipid research*, *56*(7), 1245-1247.

Distefano, A. M., GARCÍA-MATA, C. A. R. L. O. S., Lamattina, L., & Laxalt, A. M. (2008). Nitric oxide-induced phosphatidic acid accumulation: a role for phospholipases C and D in stomatal closure. *Plant, cell & environment, 31*(2), 187-194.

Do Heo, W., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., & Meyer, T. (2006). PI (3, 4, 5) P3 and PI (4, 5) P2 lipids target proteins with polybasic clusters to the plasma membrane. *Science*, *314*(5804), 1458-1461.

Fadeel, B., & Xue, D. (2009). The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. *Critical reviews in biochemistry and molecular biology*, *44*(5), 264-277.

Fantini, J., Garmy, N., Mahfoud, R., & Yahi, N. (2002). Lipid rafts: structure, function and role in HIV, Alzheimer's and prion diseases. *Expert reviews in molecular medicine, 4*(27), 1-22

Footitt, S., Dietrich, D., Fait, A., Fernie, A. R., Holdsworth, M. J., Baker, A., & Theodoulou, F. L. (2007). The COMATOSE ATP-binding cassette transporter is required for full fertility in Arabidopsis. *Plant physiology*, *144*(3), 1467-1480.

Gall, W. E., Geething, N. C., Hua, Z., Ingram, M. F., Liu, K., Chen, S. I., & Graham, T. R. (2002). Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. *Current biology*, *12*(18), 1623-1627.

Gan, P. H., Rafiqi, M., Ellis, J. G., Jones, D. A., Hardham, A. R., & Dodds, P. N. (2010). Lipid binding activities of flax rust AvrM and AvrL567 effectors. *Plant signaling & behavior*, *5*(10), 1272-1275.

Gault, C. R., Obeid, L. M., & Hannun, Y. A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. In *Sphingolipids as Signaling and Regulatory Molecules* (pp. 1-23). Springer, New York, NY.

Geertsma, E. R., & Dutzler, R. (2011). A versatile and efficient high-throughput cloning tool for structural biology. *Biochemistry*, *50*(15), 3272-3278.

Geertsma, E. R., Mahmood, N. N., Schuurman-Wolters, G. K., & Poolman, B. (2008). Membrane reconstitution of ABC transporters and assays of translocator function. *Nature protocols*, *3*(2), 256.

Goepfert, S., & Poirier, Y. (2007). β-Oxidation in fatty acid degradation and beyond. *Current opinion in plant biology, 10*(3), 245-251.

Gohil, V. M., Hayes, P., Matsuyama, S., Schägger, H., Schlame, M., & Greenberg, M. L. (2004). Cardiolipin biosynthesis and mitochondrial respiratory chain function are interdependent. *Journal of Biological Chemistry*, *279*(41), 42612-42618.

Govrin, E. M., & Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. *Current biology*, *10*(13), 751-757.

Graham, T. R. (2004). Flippases and vesicle-mediated protein transport. *Trends in cell biology*, *14*(12), 670-677.

Hanson, C. J., Bootman, M. D., & Roderick, H. L. (2004). Cell signalling: IP3 receptors channel calcium into cell death. *Current Biology*, *14*(21), R933-R935.

Harding, C., Heuser, J., & Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *The Journal of cell biology*, *97*(2), 329-339.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annual review of cell biology*, *8*(1), 67-113.

Higgins, C. F. (2001). ABC transporters: physiology, structure and mechanism–an overview. *Research in microbiology*, *152*(3-4), 205-210.

Hua, Z., Fatheddin, P., & Graham, T. R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Molecular biology of the cell*, *13*(9), 3162-3177.

Hulbert, S. H., Bai, J., Fellers, J. P., Pacheco, M. G., & Bowden, R. L. (2007). Gene expression patterns in near isogenic lines for wheat rust resistance gene Lr34/Yr18. *Phytopathology*, *97*(9), 1083-1093.

Irvine, R. F. (1982). How is the level of free arachidonic acid controlled in mammalian cells?. *Biochemical Journal*, *204*(1), 3.

Irving, H. R., Gehring, C. A., & Parish, R. W. (1992). Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proceedings of the National Academy of Sciences, 89*(5), 1790-1794.

Jambunathan, N., Siani, J. M., & McNellis, T. W. (2001). A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. *The Plant Cell, 13*(10), 2225-2240.

Kaiser, W. M. (1982). Correlation between changes in photosynthetic activity and changes in total protoplast volume in leaf tissue from hygro-, meso-and xerophytes under osmotic stress. *Planta, 154*(6), 538-545.

Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., ... & Lawrence, C. B. (2010). External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell*, *142*(2), 284-295.

Kang, J., Hwang, J. U., Lee, M., Kim, Y. Y., Assmann, S. M., Martinoia, E., & Lee, Y. (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proceedings of the National Academy of sciences*, *107*(5), 2355-2360.

Kates, M., Pugh, E. L., & Ferrante, G. (1984). Regulation of membrane fluidity by lipid desaturases. In *Membrane fluidity* (pp. 379-395). Springer, Boston, MA.

Klappe, K., Hummel, I., Hoekstra, D., & Kok, J. W. (2009). Lipid dependence of ABC transporter localization and function. *Chemistry and Physics of Lipids, 161*(2), 57-64.

Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., & Maeshima, M. (2006). Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. *Plant and Cell Physiology, 47*(3), 309-318.

Kobayashi, K., Nakamura, Y., & Ohta, H. (2009). Type A and type B monogalactosyldiacylglycerol synthases are spatially and functionally separated in the plastids of higher plants. *Plant Physiology and Biochemistry*, *47*(6), 518-525.

Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M., & Iba, K. (1994). Genetic enhancement of cold tolerance by expression of a gene for chloroplast [omega]-3 fatty acid desaturase in transgenic tobacco. *Plant Physiology*, *105*(2), 601-605.

Kohlwein, S. D., Veenhuis, M., & van der Klei, I. J. (2013). Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat—store'em up or burn'em down. *Genetics, 193*(1), 1-50.

Köster, W. (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Research in microbiology*, *152*(3-4), 291-301.

Krattinger, S. G., Lagudah, E. S., Spielmeyer, W., Singh, R. P., Huerta-Espino, J., McFadden, H., ... & Keller, B. (2009). A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science*, *323*(5919), 1360-1363.

Krattinger, S. G., Sucher, J., Selter, L. L., Chauhan, H., Zhou, B., Tang, M., ... & Schaffrath, U. (2016). The wheat durable, multipathogen resistance gene Lr34 confers partial blast resistance in rice. *Plant biotechnology journal, 14*(5), 1261-1268.

Krishnamurthy, S., Dixit, B. L., Gupta, C. M., Milewski, S., & Prasad, R. (2002). ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. *Yeast, 19*(4), 303-318.

Kunau, W. H., & Dommes, P. (1978). Degradation of unsaturated fatty acids. *The FEBS Journal*, *91*(2), 533-544.

Kuromori, T., Miyaji, T., Yabuuchi, H., Shimizu, H., Sugimoto, E., Kamiya, A., ... & Shinozaki, K. (2010). ABC transporter AtABCG25 is involved in abscisic acid transport and responses. *Proceedings of the National Academy of Sciences*, *107*(5), 2361-2366.

Laub, K. R., Marek, M., Stanchev, L. D., Herrera, S. A., Kanashova, T., Bourmaud, A., ... & Pomorski, T. G. (2017). Purification and characterisation of the yeast plasma membrane ATP binding cassette transporter Pdr11p. *PloS one, 12*(9), e0184236.

Lev, S. (2010). Non-vesicular lipid transport by lipid-transfer proteins and beyond. Nature reviews *Molecular cell biology, 11*(10), 739-750.

Liang, H., Yao, N., Song, J. T., Luo, S., Lu, H., & Greenberg, J. T. (2003). Ceramides modulate programmed cell death in plants. *Genes & development*, *17*(21), 2636-2641.

Locher, K. P. (2016). Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nature structural & molecular biology, 23*(6), 487-493.

Lodish H, Berk A, Zipursky SL, et al. (2000). Molecular Cell Biology. 4th edition. New York: W. H. Freeman;. Section 17.3, Overview of the Secretory Pathway

Lomasney, J. W., Cheng, H. F., Roffler, S. R., & King, K. (1999). Activation of phospholipase C δ1 through C2 domain by a Ca^{2+}-enzyme-phosphatidylserine ternary complex. *Journal of Biological Chemistry*, *274*(31), 21995-22001.

Lopéz-Marqués, R. L., Poulsen, L. R., & Palmgren, M. G. (2012). A putative plant aminophospholipid flippase, the *Arabidopsis* P4 ATPase ALA1, localizes to the plasma membrane following association with a β-subunit. *PLoS One*, *7*(4), e33042.

Lopéz-Marqués, R. L., Poulsen, L. R., Hanisch, S., Meffert, K., Buch-Pedersen, M. J., Jakobsen, M. K., ... & Palmgren, M. G. (2010). Intracellular targeting signals and lipid specificity determinants of the ALA/ALIS P4-ATPase complex reside in the catalytic ALA α-subunit. *Molecular biology of the cell*, *21*(5), 791-801.

McDowell, S. C., Lopez-Marques, R. L., Poulsen, L. R., Palmgren, M. G., & Harper, J. F. (2013). Loss of the *Arabidopsis thaliana* P4-ATPase ALA3 reduces adaptability to temperature stresses and impairs vegetative, pollen, and ovule development. *PloS one*, *8*(5), e62577.

Mekhedov, S., de Ilárduya, O. M., & Ohlrogge, J. (2000). Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis. *Plant Physiology, 122(2)*, 389-402.

Michaelson, L. V., Napier, J. A., Molino, D., & Faure, J. D. (2016). Plant sphingolipids: Their importance in cellular organization and adaption. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1861*(9), 1329-1335.

Mishima, K., Nakajima, M., & Ogihara, T. (2004). Effects of lysophospholipids on membrane order of phosphatidylcholine. *Colloids and Surfaces B: Biointerfaces*, *33*(3-4), 185-189.

Molday, R. S., Zhong, M., & Quazi, F. (2009). The role of the photoreceptor ABC transporter ABCA4 in lipid transport and Stargardt macular degeneration. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1791*(7), 573-583.

Moritz, A., De Graan, P. N., Gispen, W. H., & Wirtz, K. W. (1992). Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *Journal of Biological Chemistry, 267*(11), 7207-7210.

Munnik, T. (2001). Phosphatidic acid: an emerging plant lipid second messenger. *Trends in plant science*, *6*(5), 227-233.

Munnik, T., Irvine, R. F., & Musgrave, A. (1998). Phospholipid signalling in plants. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1389*(3), 222-272.

Murray, D., & Honig, B. (2002). Electrostatic control of the membrane targeting of C2 domains. *Molecular cell, 9*(1), 145-154.

Nagata, T., Nemoto, Y., & Hasezawa, S. (1992). Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *International Review of Cytology, 132*, 1-30.

Newton, A. C. (1995). Protein kinase C: structure, function, and regulation. *Journal of Biological Chemistry*, *270*(48), 28495-28498.

Ng, C. K. Y., Carr, K., McAinsh, M. R., Powell, B., & Hetherington, A. M. (2001). Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. *Nature*, *410*(6828), 596.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., ... & Schweizer, P. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. *The Plant Cell*, *22*(9), 3130-3141.

O'Brien, I. E., Reutelingsperger, C. P., & Holdaway, K. M. (1997). Annexin-V and TUNEL use in monitoring the progression of apoptosis in plants. *Cytometry Part A*, *29*(1), 28-33.

Ohlrogge, J., & Browse, J. (1995). Lipid biosynthesis. *The Plant Cell*, *7*(7), 957.

Olivera, A., Rosenthal, J., & Spiegel, S. (1996). Effect of acidic phospholipids on sphingosine kinase. *Journal of cellular biochemistry*, *60*(4), 529-537.

Özvegy, C., Litman, T., Szakács, G., Nagy, Z., Bates, S., Váradi, A., & Sarkadi, B. (2001). Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochemical and biophysical research communications*, *285*(1), 111-117.

Pappan, K., Austin-Brown, S., Chapman, K. D., & Wang, X. (1998). Substrate selectivities and lipid modulation of plant phospholipase Dα,-β, and-γ. *Archives of biochemistry and biophysics, 353*(1), 131-140.

Petschnigg, J., Wolinski, H., Kolb, D., Zellnig, G., Kurat, C. F., Natter, K., & Kohlwein, S. D. (2009). Good fat, essential cellular requirements for triacylglycerol synthesis to maintain membrane homeostasis in yeast. *Journal of Biological Chemistry, 284*(45), 30981-30993.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., & Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nature chemical biology*, *5*(5), 308.

Pighin, J. A., Zheng, H., Balakshin, L. J., Goodman, I. P., Western, T. L., Jetter, R., ... & Samuels, A. L. (2004). Plant cuticular lipid export requires an ABC transporter. *Science, 306*(5696), 702-704.

Posor, Y., Eichhorn-Gruenig, M., Puchkov, D., Schöneberg, J., Ullrich, A., Lampe, A., ... & Krauss, M. (2013). Spatiotemporal control of endocytosis by phosphatidylinositol-3, 4-bisphosphate. *Nature*, *499*(7457), 233.

Poulsen, L. R., López-Marqués, R. L., McDowell, S. C., Okkeri, J., Licht, D., Schulz, A., ... & Palmgren, M. G. (2008). The *Arabidopsis* P4-ATPase ALA3 localizes to the Golgi and requires a β-subunit to function in lipid translocation and secretory vesicle formation. *The Plant Cell*, *20*(3), 658-676.

Poulsen, L. R., López-Marqués, R. L., Pedas, P. R., McDowell, S. C., Brown, E., Kunze, R., ... & Palmgren, M. (2015). A phospholipid uptake system in the model plant *Arabidopsis thaliana*. *Nature communications, 6*, 7649.

Rajasekharan, A., & Gummadi, S. N. (2011). Flip-flop of phospholipids in proteoliposomes reconstituted from detergent extract of chloroplast membranes: Kinetics and phospholipid specificity. *PloS one, 6*(12), e28401.

Rebecchi, M., Boguslavsky, V., Boguslavsky, L., & McLaughlin, S. (1992). Phosphoinositide-specific phospholipase C-. delta. 1: effect of monolayer surface pressure and electrostatic surface potentials on activity. *Biochemistry, 31*(51), 12748-12753.

Resh, M. D. (2013). Covalent lipid modifications of proteins. *Current biology*, *23*(10), R431-R435.

Rinaldo, A., Gilbert, B., Boni, R., Krattinger, S. G., Singh, D., Park, R. F., ... & Ayliffe, M. (2017). The Lr34 adult plant rust resistance gene provides seedling resistance in durum wheat without senescence. *Plant biotechnology journal*, *15*(7), 894-905.

Risk, J. M., Selter, L. L., Chauhan, H., Krattinger, S. G., Kumlehn, J., Hensel, G., ... & Lagudah, E. S. (2013). The wheat Lr34 gene provides resistance against multiple fungal pathogens in barley. *Plant biotechnology journal, 11*(7), 847-854.

Sabeva, N. S., Liu, J., & Graf, G. A. (2009). The ABCG5 ABCG8 sterol transporter and phytosterols: implications for cardiometabolic disease. *Current opinion in endocrinology, diabetes, and obesity, 16*(2), 172.

Schnippenkoetter, W., Lo, C., Liu, G., Dibley, K., Chan, W. L., White, J., ... & Godwin, I. (2017). The wheat Lr34 multipathogen resistance gene confers resistance to anthracnose and rust in sorghum. *Plant biotechnology journal*, *15*(11), 1387-1396.

Segawa, K., Kurata, S., Yanagihashi, Y., Brummelkamp, T. R., Matsuda, F., & Nagata, S. (2014). Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science*, *344*(6188), 1164-1168.

Singh, H., Derwas, N., & Poulos, A. (1987). Very long chain fatty acid β-oxidation by rat liver mitochondria and peroxisomes. *Archives of biochemistry and biophysics*, *259*(2), 382-390.

Solovchenko, A. E. (2012). Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. *Russian Journal of Plant Physiology*, *59*(2), 167-176.

Spiegel, S., Foster, D., & Kolesnick, R. (1996). Signal transduction through lipid second messengers. *Current opinion in cell biology*, *8*(2), 159-167.

Stace, C. L., & Ktistakis, N. T. (2006). Phosphatidic acid-and phosphatidylserine-binding proteins. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1761*(8), 913-926.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B. H., Molina, A., Schulze-Lefert, P., ... & Somerville, S. (2006). *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *The Plant Cell, 18*(3), 731-746.

Storz, P., & Toker, A. (2002). 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. *Front Biosci*, *7*, d886-902.

Subczynski, W. K., Hyde, J. S., & Kusumi, A. (1991). Effect of alkyl chain unsaturation and cholesterol intercalation on oxygen transport in membranes: a pulse ESR spin labeling study. *Biochemistry*, *30*(35), 8578-8590.

Subramaniam, R., Després, C., & Brisson, N. (1997). A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato. *The Plant Cell*, *9*(4), 653-664.

Sucher, J., Boni, R., Yang, P., Rogowsky, P., Büchner, H., Kastner, C., ... & Keller, B. (2017). The durable wheat disease resistance gene Lr34 confers common rust and northern corn leaf blight resistance in maize. *Plant biotechnology journal, 15*(4), 489-496.

Tarling, E. J., de Aguiar Vallim, T. Q., & Edwards, P. A. (2013). Role of ABC transporters in lipid transport and human disease. *Trends in Endocrinology & Metabolism, 24*(7), 342-350.

Thompson, J. R., Register, E., Curotto, J., Kurtz, M., & Kelly, R. (1998). An improved protocol for the preparation of yeast cells for transformation by electroporation. *Yeast, 14*(6), 565-571.

Tomsig, J. L., & Creutz, C. E. (2000). Biochemical characterization of copine: a ubiquitous Ca2+-dependent, phospholipid-binding protein. *Biochemistry*, *39*(51), 16163-16175.

Toussaint, F., Pierman, B., Bertin, A., Lévy, D., & Boutry, M. (2017). Purification and biochemical characterization of NpABCG5/NpPDR5, a plant pleiotropic drug resistance transporter expressed in *Nicotiana tabacum* BY-2 suspension cells. *Biochemical Journal, 474*(10), 1689-1703.

Tucker, S. L., & Talbot, N. J. (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annual review of phytopathology*, *39*(1), 385-417.

Van Anthony, M. V., Cuevas, S., Zheng, X., & Jose, P. A. (2016). Localization and signaling of GPCRs in lipid rafts. *Methods in cell biology, 132,* 3-23

Van Meer, G., Voelker, D. R., & Feigenson, G. W. (2008). Membrane lipids: where they are and how they behave. *Nature reviews Molecular cell biology*, *9*(2), 112.

Von Zastrow, M., & Kobilka, B. K. (1992). Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *Journal of Biological Chemistry*, *267*(5), 3530-3538.

Wang, X. (2005). Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. *Plant Physiology, 139*(2), 566-573.

Wang, X., Wang, C., Sang, Y., Qin, C., & Welti, R. (2002). Networking of phospholipases in plant signal transduction. *Physiologia Plantarum*, *115*(3), 331-335.

Wang, X., Zhang, W., Li, W., & Mishra, G. (2007). Phospholipid signaling in plant response to drought and salt stress. In Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops (pp. 183-192). *Springer, Dordrecht.*

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., & Goodenough, U. (2009). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryotic cell*, *8*(12), 1856-1868.

Wang, J., Ding, B., Guo, Y., Li, M., Chen, S., Huang, G., & Xie, X. (2014). Overexpression of a wheat phospholipase D gene, TaPLDα, enhances tolerance to drought and osmotic stress in *Arabidopsis thaliana. Planta, 240*(1), 103-115.

Weingärtner, A., Kemmer, G., Müller, F. D., Zampieri, R. A., dos Santos, M. G., Schiller, J., & Pomorski, T. G. (2012). *Leishmania* promastigotes lack phosphatidylserine but bind annexin V upon permeabilization or miltefosine treatment. *PloS one, 7*(8), e42070.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., ... & Hein, I. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature*, *450*(7166), 115.

Wiesinger, C., Kunze, M., Regelsberger, G., Forss-Petter, S., & Berger, J. (2013). Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction. *Journal of Biological Chemistry, 288*(26), 19269-19279.

Wirthmueller, L., Maqbool, A., & Banfield, M. J. (2013). On the front line: structural insights into plant-pathogen interactions. *Nature Reviews Microbiology, 11*(11), 761-776.

Woehlecke, H., Antje, P. O. H. L., Alder-Baerens, N., Hermann, L. A. G. E., & Herrmann, A. (2003). Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2). *Biochemical Journal, 376*(2), 489-495.

Wu, J. X., Li, J., Liu, Z., Yin, J., Chang, Z. Y., Rong, C., ... & Yao, N. (2015). The *Arabidopsis* ceramidase AtACER functions in disease resistance and salt tolerance. *The Plant Journal*, *81*(5), 767-780.

Xie, L. J., Chen, Q. F., Chen, M. X., Yu, L. J., Huang, L., Chen, L., ... & Yin, J. (2015). Unsaturation of very-long-chain ceramides protects plant from hypoxia-induced damages by modulating ethylene signaling in *Arabidopsis*. *PLoS genetics*, *11*(3), e1005143.

Xue, H., Chen, X., & Li, G. (2007). Involvement of phospholipid signaling in plant growth and hormone effects. *Current opinion in plant biology, 10*(5), 483-489.

Yaeno, T., Li, H., Chaparro-Garcia, A., Schornack, S., Koshiba, S., Watanabe, S., ... & Shirasu, K. (2011). Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. *Proceedings of the National Academy of Sciences*, *108*(35), 14682-14687.

Yamaguchi, T., Kuroda, M., Yamakawa, H., Ashizawa, T., Hirayae, K., Kurimoto, L., ... & Shibuya, N. (2009). Suppression of a phospholipase D gene, *Os*PLDβ1, activates defense responses and increases disease resistance in rice. *Plant Physiology*, *150*(1), 308-319.

Yang, D. L., Shi, Z., Bao, Y., Yan, J., Yang, Z., Yu, H., ... & Xu, D. (2017). Calcium pumps and interacting BON1 protein modulate calcium signature, stomatal closure, and plant immunity. *Plant physiology*, *175*(1), 424-437.

Zachowski, A. (1993). Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochemical Journal*, *294*(Pt 1), 1.

Zechner, R., Strauss, J. G., Haemmerle, G., Lass, A., & Zimmermann, R. (2005). Lipolysis: pathway under construction. *Current opinion in lipidology*, *16*(3), 333-340.

Zhang, W., Qin, C., Zhao, J., & Wang, X. (2004). Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(25), 9508-9513.

Zhao, J. (2015). Phospholipase D and phosphatidic acid in plant defence response: from protein–protein and lipid–protein interactions to hormone signalling. *Journal of Experimental Botany*, *66*(7), 1721-1736.

Zhao, J., Devaiah, S. P., Wang, C., Li, M., Welti, R., & Wang, X. (2013). *Arabidopsis* phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytologist*, *199*(1), 228-240.

Zimorski, V., Ku, C., Martin, W. F., & Gould, S. B. (2014). Endosymbiotic theory for organelle origins. *Current opinion in microbiology*, *22*, 38-48.

Zou, B., Hong, X., Ding, Y., Wang, X., Liu, H., & Hua, J. (2016). Identification and analysis of copine/BONZAI proteins among evolutionarily diverse plant species. *Genome, 59*(8), 565-573.

Zvereva, A. S., & Pooggin, M. M. (2012). Silencing and innate immunity in plant defense against viral and non-viral pathogens. *Viruses, 4*(11), 2578-2597.

8 Acknowledgements

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